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STRAIN VARIABILITY OF WESTERN CANADIAN ISOLATES OF *LEPTOSPHAERIA MACULANS*, THE CAUSAL AGENT OF BLACKLEG DISEASE OF CANOLA.

by

©

RALPH MARTIN LANGE

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF **MASTER OF SCIENCE**

IN

PLANT PATHOLOGY

DEPARTMENT OF PLANT SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **STRAIN VARIABILITY OF WESTERN CANADIAN ISOLATES OF *LEPTOSPHAERIA MACULANS*, THE CAUSAL AGENT OF BLACKLEG DISEASE OF CANOLA.** submitted by **RALPH MARTIN LANGE** in partial fulfillment of the requirements for the degree of **Master of Science** in **PLANT PATHOLOGY**.

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Date: 27 Aug/93

Abstract

Leptosphaeria maculans, the causal agent of blackleg of canola (*Brassica napus* and *B. rapa*) occurs as distinct aggressive and non-aggressive strains. Recently, host specificity at the cultivar level has been observed among isolates of the aggressive strain. This specialization of the fungus for differing host genetic backgrounds suggests that more than one pathotype exists within the *L. maculans* population. The number of pathotypes will influence the choice of strategies used to incorporate blackleg resistance into *Brassica* backgrounds. The objectives of this research were to characterize the variability in aggressivity and selected cultural characteristics of western Canadian isolates of *L. maculans*. An additional objective was to associate host specificity of the fungus with variation among isolates in cell-surface carbohydrates, based on the hypothesis that these moieties condition host-pathogen recognition.

Of the 67 isolates collected in Alberta, Saskatchewan and Manitoba, 45 and 22 were classed as aggressive and non-aggressive, respectively, based on interaction phenotypes resulting from inoculation onto *B. napus* cotyledons. None of the western Canadian isolates were able to cause severe lesions on cotyledons of the differential *B. napus* cvs. Glacier and Quinta, indicating that these isolates belong to a single pathotype. Virulence of *L. maculans* isolates was also assessed on *B. napus* cv. Maluka. While non-aggressive isolates caused slight necrosis only, aggressive isolates produced a range of symptoms when Maluka cotyledons were challenged.

In culture, non-aggressive isolates generally had significantly greater colony expansion and germ-tube extension rates than aggressive isolates. Colonies of most non-aggressive isolates were morphologically distinct from those of aggressive isolates. Quantification of *in vitro* pigment production by spectrophotometry and image analysis showed that non-aggressive isolates produced significantly greater amounts of pigment than aggressive isolates.

Washed *L. maculans* conidiospores failed to agglutinate when suspended in a set of lectin solutions, although washed rat erythrocytes and *Fusarium avenaceum* microconidia were agglutinated. Therefore, no variation among isolates with respect to cell-surface carbohydrates was detected.

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Chapter 1

Literature review.

Introduction

The Brassicaceae is a large and varied family of flowering plants that includes many domesticated species. The name is descriptive of the flowers, which are composed of four sepals, four petals in the shape of a cross, and six stamens, the outer two of which are inserted lower than the inner four (Moss 1983, Prakesh & Hinata 1980). All members of the family produce siliques or silicles, fruits which are unique to the family (Stern 1982).

Many of the domesticated members of the Brassicaceae are important agricultural species, particularly in cool climates. A number of common leaf and root vegetables and condiments are crucifers. Other agriculturally important cruciferous plants include dye plants (dyer's woad) and plants used as industrial oil sources for the manufacture of soap and lamp oils (*Camelina sativa*) (Stern 1982). Many crucifers are wild or weedy species. *Armoracia rusticana*, the source of horseradish peroxidase, and *Arabidopsis thaliana*, which has been used as a model system in many physiolgical and molecular biological studies, are also members of the family. Four cruciferous species *Brassica napus*., *B. rapa* (syn. *B. campestris*), *B. juncea* and *B. carinata* are among the world's most important sources of edible oils (Rimmer & van den Berg 1992). In Canada, the largest source of edible oil is canola (Bergh 1991). Canola is defined as rapeseed with less than 2% erucic acid in the seed oil, and have 30 μmol g^{-1} or less of aliphatic glucosinolates in the meal. Canola accounts for 63% of the vegetable oil and 82% of the salad oils consumed in Canada (Bergh 1991).

Two species, *B. napus* and *B. rapa*, are among the most important crops in Canada, both in terms of production (Anon. 1991a) and crop value (Bergh 1991), and are certainly the most important oilseeds. The total area seeded to canola (*B. napus* and *B. rapa*) in Canada in the 1990-1991 crop year was 3.268 million hectares, an area which produced 6.4 million tonnes of seed. Argentine canola (*B. napus*) cultivars accounted for 55.9% of the area seeded to canola in Canada in 1989, with Polish rape (*B. rapa*) cultivars composing 43.6% (De Clercq et al. 1989). Wheat production in the same period was 32.8 million tonnes, while the total production of grain corn, oats, rye and mixed grains was 22.7 million tonnes (Bergh 1991). The canola oil processing industry is also important to the country. The direct economic benefit of oil and meal processing in Canada in 1991 has been estimated at C$1,575 million, with an additional contribution to the balance of payments through import replacement and export earnings of C$469 and C$207 million, respectively (Bergh 1991).

Brassica napus is an ancient crop. Root crop forms (Swedes) were known in antiquity, but oleiferous forms are not mentioned in Greek or Roman literature (Downey 1983, Prakash & Hinata 1980). Wild forms of *B. napus* have not been found, and the species is almost certainly the result of hybridization between *B. rapa* and *B. oleracea* .

Interspecific hybridization is not unusual in *Brassica*. It has long been known that three species, *B. carinata*, *B. juncea* and *B. napus*, are amphidiploids derived from crosses between the monogenomic or basic species *B. nigra*, *B. oleracea* and *B. rapa* (Downey & Röbbelen 1989; Prakash & Hinata 1980; U 1934, 1935). The relationships between these species were elucidated through analysis of chromosome conjugation in progeny of interspecific crosses (Downey 1983). Hybridization of monogenomic *Brassica* species has resulted in artificial synthesis of *B. napus* (U 1935), *B. juncea* and *B. carinata* (Downey 1983, Rimmer & van den Berg 1992). These and other studies (Vaughn 1977, Verma & Rees 1974) have confirmed the amphidiploid origin of *B. napus*, *B. juncea* and *B. carinata*. The letter designations A, B and C have been assigned to the basic species *B. rapa*, *B. nigra* and *B. oleracea*, respectively (Prakash & Hinata, 1980). Thus, for example, *B. napus* may be described by the formula aacc. The amphidiploid *Brassica* species all form bivalents between homologous chromosomes at meiosis (Prakash & Hinata 1980). The cytogenetic interrelationships among these species is summarized by the triangle of U (1935) (Figure 1.1).

Blackleg is an important disease of oleiferous *Brassica napus*, as well as of other cruciferous crops (Dixon 1981, Gugel & Petrie 1992). The causal agent, *Leptosphaeria maculans* (Desm.) Ces. *et* de Not. is an ascomycetous fungus of the order Pleosporales. Its anamorph is *Phoma lingam* (Tode *ex* Fr.) Desm., a deuteromycete of the order Sphaeropsidales (Agrios, 1988). *Phoma lingam* was first described by Tode in 1791 as a saprophyte of cabbage and was named *Sphaeria lingam* (Pound 1947). The fungus was transferred to the genus

Phoma in 1849 by Desmaziéres, who isolated it from living plants (Pound 1947). The fungus has also been named *Phyllosticta brassica* Westend. (Punithalingam & Holliday 1972). Fungi which appear to be synonymous with *Phoma lingam* are *P. brassicae* (Thüm.) Sacc. and *Phoma lingam* var *napobrassica* (Rostrup) Grove (Smith & Sutton 1964).

Boerema & van Kesteren (1964) challenged the validity of the name *Phoma lingam* because of substantial differences from the form genus *Phoma* Sacc. and concluded that the correct name for the pycnidial stage of *Leptosphaeria maculans* is *Plenodomus lingam* (Tode *ex* Fr.) Höhn. However, the name *Phoma lingam* has been in general use since 1849, hence the binomial has been retained (Ndimande 1976).

Synonyms for the ascogenous state are *Sphaeria maculans* (Desm.) and *Pleospora maculans* (Desm.) Tul. The fungus *Leptosphaeria napi* (Fuckel) Sacc. is apparently identical to *L. maculans*. (Punithalingam & Holliday, 1972).

The pathogen has been reported in 49 countries and on all continents except Antarctica (Punithalingam & Holliday 1972). In Canada, the fungus is found in almost all areas where *B. napus* or B. rapa are grown. In Alberta the fungus is largely confined to areas east and south of Edmonton (Evans et al., 1990, 1991, 1992; Kharbanda et al., 1988, 1989). Evans et al. (1991) estimated the 1991 province-wide yield loss to be 1% of the total canola production of that year, with 85 of 293 fields surveyed showing trace to 5% disease incidence levels. An extensive blackleg of canola survey in the county of Flagstaff found virulent blackleg in 49.9% of the fields examined (Evans et al. 1992). The disease was absent from the Peace River region of Alberta and British Columbia (Buonassisi & MacDonald 1990; Harrison 1988, 1989, 1990, 1992; Harrison & Loland 1991, MacDonald 1992) until 1992, when the virulent strain of *L. maculans* was found in a single field in the Alberta portion of the region (Evans et al. 1993).

The disease is a serious yield limiting factor in central Saskatchewan (Petrie 1986a). It has been increasing in all other canola-growing regions of the province since its initial discovery in 1976 (McGee & Petrie 1978, Petrie 1978), and particularly since 1986 (Berkenkamp & Kirkham 1988, 1989, 1991; Jesperson 1990; Kirkham & Berkenkamp 1990). In 1991, blackleg was found in 76% of fields surveyed, and was probably responsible for major yield losses (Kirkham 1992).

Blackleg was found in 41 of 84 fields surveyed in Manitoba in 1990 (van den Berg & Platford 1991). In 1984, the pathogen was first discovered in the south-west region of the province, near Souris (Platford 1985) The disease has since spread throughout the province, with the highest disease incidences being found in the western region of the province (Platford 1988, Platford & van den Berg 1989, van den Berg & Platford 1991, van den Berg et al. 1992).

Disease severity in Ontario is high, given the relatively limited area seeded to canola. The disease became especially serious when producers began seeding larger areas of winter-type cultivars of *B. napus* (Gugel & Petrie 1992, Peters & Hall 1987).

Blackleg is an important disease worldwide. The disease has been especially important in Australia, where a general epidemic occurred in 1972 following generalized outbreaks in 1971 in all rapeseed-growing areas of the country (McGee & Emmett 1977, Wood & Barbetti 1977b). This resulted in a 96% decrease in the area of canola sown by 1974 (Roy & Reeves 1975).

The disease has been severe in Europe in the past, although it is now effectively controlled by genetic resistance (Rimmer & van den Berg 1992). The disease became important in Germany after the introduction of low-erucic acid *B. napus* cultivars (Krüger 1982, 1983). The disease has also been periodically serious in England (Gladders & Musa 1980, Humpherson-Jones 1983b), and epidemics have occurred in France in 1950 and 1966 (Gugel & Petrie 1992).

It is clear, therefore, that *L. maculans* represents an important threat to production of edible oil from oleiferous *B. napus* and *B. rapa* cultivars. The pathogen has received a great deal of attention since early in this century (Henderson 1918), and particularly since the development of low-erucic, low-glucosinolate *B. napus* and *B. rapa* cultivars.

Disease cycle, signs and symptoms

The blackleg disease cycle often begins with the introduction of infested seed into a previously unaffected field. Mycelium survives for long periods under the seed coats of *Brassica* species (Gabrielson 1983). Such seeds may germinate to produce infected seedlings. Pycnidia of the anamorph form on cotyledons and hypocotyls, producing conidia which are primarily distributed by rain-splash (Hall 1992). These conidia, and those produced as a result of subsequent infections, serve as secondary inoculum. Lesions may develop on leaves, stems and pods. The pathogen survives in mycelial form with the lignified host residues remaining after senescence. Pseudothecia develop on rape and other cruciferous residue early in spring, producing ascospores which may be effectively dispersed by wind over a few kilometers (Gugel & Petrie 1992, Hall 1992). *Leptosphaeria maculans* may also survive winter as mycelium in biennial cultivars of *B. napus*, becoming active once temperatures increase in spring (Nathaniels & Taylor 1983).

Stem and crown cankers are the most destructive manifestations of the disease in oilseed rape, particularly when infections are initiated by virulent *L. maculans* before the six-leaf stage (McGee & Petrie 1978). Severe infections result in girdling, stem splitting, wilting and lodging. Cankers restrict the flow of nutrients and moisture within the conductive tissues in the stem, causing premature ripening (Gugel & Petrie 1992).

Initially lesions are indefinite and may appear water-soaked. Tissue within the lesion may be collapsed. Stem lesions become tan to brown oblong sunken cankers which often have a dark purple border. Cracks may develop within or near cankers (Figure 1.2). Crown cankers may extend below the soil surface, where they take on a brownish hue. Stems of infected plants are often blackened internally (Newman & Bailey, 1987).

The pathogen also attacks cotyledons and leaves of *B. napus* and *B. rapa* (Gabrielson 1983). These lesions are similar in appearance to stem cankers, except that they can be of various shapes. *Leptosphaeria maculans* may also cause damping-off symptoms, which result from the fungus killing the seedlings before or shortly after emergence (Hall 1992).

Mature lesions contain numerous erumpent black pycnidia, the asexual fruiting structure of the fungus. Two types of pycnidia are produced. Type I are formed on living tissue and are light colored, thin walled and have narrow ostioles. Type II pycnidia are produced on woody or dead host tissue. These are globose, black and thick-walled (Gabrielson, 1983; Henderson, 1918; Punithalingam & Holliday, 1972). Pycnidia emit large numbers of unicellular hyaline oblong conidia (3-5 × 1.5-2 µm) under conditions of high humidity. The exuded spore masses have a pink to purple appearance (Martens et al. 1984, Punithalingam & Holliday 1972).

The telomorphic state of the pathogen (*L. maculans*) begins to form sexual fruiting structures, pseudothecia, on basal stems late in the growing season (Gladders & Musa 1980). Ascocarps (Figure 1.3) are also produced on stem and leaf debris beginning one year after initial infection and continuing up to five years thereafter (Anonymous 1987; Gabrielson 1983; Gladders & Musa 1980; Humpherson-Jones 1983a, 1983b; Martens *et al.* 1985; McGee & Petrie 1979). Pseudothecia are erumpent, black, globose and may have protruding ostioles The yellow-brown ascospores are cylindrical to ellipsoidal, are usually 5-septate and have rounded ends. Eight ascospores are carried in each cylindrical ascus. Filiform, hyaline, septate pseudoparaphysis are formed (Punithalingam & Holliday 1972).

Leptosphaeria maculans may also infect pods and seed (Petrie & Vanterpool 1974). Infected pods may senesce and dehisce prematurely. Lesions bearing pycnidia may form on pods. Seeds may be shriveled or discolored, although often no signs or symptoms are visible on seed (Gabrielson 1983).

At least two strains of *L. maculans* infect *B. napus* and *B. rapa* in western Canada. The first, which is highly virulent on *B. napus*, produces severe symptoms of the type described above. The second strain commonly isolated from *B. napus* does not cause severe basal cankers and is not considered to be a serious disease problem in *B. napus* or *B. rapa*. The two strains are difficult to differentiate on the basis of lesion morphology, other than on the severity of symptoms they cause. Petrie (1978), however, isolated the virulent strain more frequently from dark colored lesions on stubble, whereas the weakly virulent strain was isolated more frequently from light-colored lesions.

Life cycle and genetics

The life cycle of *Leptosphaeria maculans* (Figure 1.4) is typical of many ascomycetes. No information on the mode of plasmogamy or the length of dikaryotic phase relative to other stages of the lifecycle is available in the literature. However, in many ascomycetes, ascogenous dikaryotic hyphae are formed shortly before ascus development (Burnett 1975). Some observations provide circumstantial evidence that morphologically distinct pycnidiospores emanating from multilocular pycnidia act as spermatia, since the presumed spermagonia occur only in close proximity to young pseudothecia (Smith & Sutton 1964, Ndimande 1976).

Multinucleate hyphal cells arise through hyphal fusion (Calvert & Pound 1949). No information specific to *L. maculans* is available concerning the number of nuclei in conidia or hyphal cells.

The sexual state of the pathogen may be induced in culture by layering water agar over colonies of the anamorphic state and exposing the cultures to a twelve-hour period of ultraviolet light (Williams 1992). Formation of ascocarps is limited to matings between (+) and (-) mating-type isolates of the same strain, as crosses between weakly-virulent and virulent isolates have been unsuccessful to date (Bonman et al. 1981, Ndimande 1976, Petrie & Lewis 1985, Venn 1979). This may indicate that the two strains of *L. maculans* are in fact distinct species.

Taylor et al. (1991) found substantial differences between strains with respect to DNA content and apparent chromosome number, while a high degree of similarity among isolates was found within each strain. They found that transverse alternating field electrophoresis of whole chromosomes from weakly virulent isolates produced 12-14 bands, which are estimated to represent at least 1.6×10^7 base pairs. By comparison, application of the technique to virulent isolates resulted in 6-8 bands representing a minimum of 8.7×10^6 base pairs. Using RAPD technology, Schäfer & Wöstemeyer (1992) found clear differences between aggressive and non-aggressive isolates of the pathogen. Fingerprinting of genomic (Johnson & Lewis 1990, Koch et al. 1991, Meyer et al. 1992), mitochondrial and ribosomal DNA (Johnson & Lewis 1990) revealed a far greater degree of polymorphism between *L. maculans* strains than among isolates of the same strain. These results support the hypothesis that the two *L. maculans* strains found on *B. napus* and *B. rapa* are separate species.

Control of sexual compatibility in *L. maculans* is heterothallic and bipolar, that is, it is controlled by a single diallelic gene (Venn 1979). Heterogenic incompatibility factors may exist, according to Hill & Williams (1988) since Boudart (1981) observed reduced fertility when isolates from *B. napus* and *B. oleracea* were crossed.

Hassan et al. (1991) found that extrachromosomal plasmid-like DNA was more commonly associated with aggressive isolates than with non-aggressive isolate. This observation presents the possibility that some of the genes conditioning virulence in *L. maculans* exhibit cytoplasmic inheritance. This has already been suggested for other pathosystems. For example, Kistler et al.

(1987) found that the *conglutinans* and *raphani* formae speciales of *Fusarium oxysporum* carried unique plasmid-like DNAs. Kistler et al. (1987) hypothesized that these DNAs carry genes conditioning specificity at the formae speciales, or pathotype level. Localization of genes conditioning physiological specialization in extrachromosomal DNAs may also occur in *L. maculans*, since plasmid-like DNAs are also associated with virulence in this fungus.

Parasexualism is a mode of genetic recombination wherein genetic information is exchanged between unlike nuclei within somatic cells. The sequence of events involved is; karyogamy within the heterokaryon, mitotic crossing over, and haploidization (Burnett 1975, Fincham & Day 1971). Parasexualism has been confirmed for *L. maculans* by Petrie (1969) who found that although haploidization did not occur, the resulting diploids were stable. Hence, it is probable that parasexualism supplements the sexual stage as a source of genetic variability in *L. maculans*.

Pathogenesis

Pycnidiospores and ascospores of *L. maculans* germinate under different conditions, ascospores being tolerant of a wider range of abiotic conditions than pycnidiospores, as well as being more infective (Hall 1992, Hammond & Lewis 1987b). The optimal temperature for conidiospore germination is 20° to 25° C, although germination will occur at a temperature as low as 5° C, albeit at a lower rate (Vanniasingham & Gilligan 1988). Germ tubes appear after 12 to 24 hours of incubation at 24° C (Vanniasingham & Gilligan 1988). By contrast, ascospores form within 4 to 8 hours at 4 to 28° C; low temperatures simply slowing the rate of germination and germ-tube extension (Wittern & Krüger 1985).

A number of models of initial infection of *B. napus* by *L. maculans* have been proposed. For example, *L. maculans* was at one time thought to be a soil inhabitant, and it was, therefore, hypothesized that stems were infected directly from spores splashed from the soil (Rimmer & van den Berg 1992, Barbetti 1976). Other models of initial infection include direct infection of stems via ascospore discharge from stubble (Nathaniels & Taylor 1983, Ndimande 1976, Newman 1984a, McGee & Petrie 1979), and infection of hypocotyls through wounds created by natural splitting (Nathaniels & Taylor

1983). However, Hammond & Lewis (1987b) observed that germ tubes of germinated pycnidiospores entered leaf laminae through wounds or stomata before beginning a phase of non-necrotic, intercellular growth. After penetration, *L. maculans* colonizes intercellular spaces of the spongy mesophyll until hyphae reach vascular tissue, whereupon the pioneer hyphae spread down the xylem elements or through the intercellular spaces of the xylem parenchyma and cortex (Hammond & Lewis 1987a). Thus the fungus enters into a biotrophic phase of growth. The cells surrounding the pioneer hyphae will plasmolyse in 2M sucrose, and will accumulate the vital stain sodium fluorescein, evidence that these host cells are not necrotic (Hammond & Lewis 1987b).

Leptosphaeria maculans re-enters a necrotrophic state when hyphae pass from the petiole into the stem, whereupon stem cankers are formed (Hammond & Lewis 1987b). Biotrophic growth through the lamina and petiole is not necessarily the only infection pathway, however. Cankers which developed directly from infection on the stem have been observed (Hammond & Lewis 1986). Moreover, Xi et al. (1991) observed that many infections were initiated in leaf axils.

Another significant feature of *L. maculans* pathogenesis is the ability of the fungus to cause latent infections in *B. napus* under appropriate conditions. Nathaniels & Taylor (1983) found that they could inoculate *B. napus* with the pathogen and produce plants lacking macroscopic symptoms, but with *L. maculans* hyphae in non-necrotic cortical tissue. This type of infection occurred when plants were exposed to low growth temperatures after inoculation. Nathaniels and Taylor (1983) also found that symptomless inoculated plants rapidly developed stem cankers after the plants were moved to a warm greenhouse. The latent infection phenomenon has been confirmed by the field observations of Xi et al. (1991) who showed that the fungus can be isolated from stems and petioles a few weeks before cankers become visible. This type of infection, however, may not be truly latent, since *L. maculans* hyphae are capable of growth at low temperatures (Ndimande 1976).

Phytotoxic heterocyclic amine compounds with a polysulfide bridge, called sirodesmins (Badawy & Hoppe 1989b), are produced by the fungus in vitro. These toxins were first described Curtis et al. (1977) as secondary metabolites of the fungus *Sirodesmium diversum*. At least six sirodesmins have been isolated from culture filtrates of *L. maculans*; sirodesmin PL (Férézou et al. 1977); sirodesmin B (Badawy & Hoppe 1989b); Sirodesmin C (Badawy & Hoppe 1989a, 1989b) sirodesmins H,K and J, and phomalirazine (Pedras et al. 1988, 1989; Pedras & Séguin-Swartz 1992). Structurally, the compounds differ from one another in the amount of acetylation and in the number of sulfur atoms they contain (Badawy & Hoppe 1989b, Pedras & Séguin-Swartz 1992). In addition, a number of de- and diacetyl derivatives of the sirodesmins have been isolated from *L. maculans* culture filtrates. The most commonly mentioned of these is deacetylsirodesmin PL, a compound which appears to be one of the main phytotoxins produced by the pathogen (Badawy & Hoppe 1989a, 1989b; Férézou et al. 1977; Pedras & Séguin-Swartz 1992).

The phytotoxins produced by *L. maculans* are not host-specific. Culture filtrates and purified toxins cause lesions on both host and non-host species (Badawy & Hoppe 1989a, De March et al. 1986, Pedras & Séguin-Swartz 1992). Some *Brassica* species, however, are more sensitive to *L. maculans* phytotoxins than others. The relative toxicity of *L. maculans* phytotoxins varies with host species. For example, the LD_{50} of *B. juncea* cv. Cutlass microspore cultures exposed to phomalirazine was estimated to be 1.89 μM; the equivalent LD_{50} value for sirodesmin PL was 2.99 μM (Pedras & Séguin-Swartz 1992). Microspores of *Brassica napus* cv. Westar, however, are equally susceptible to either phytotoxin (Pedras & Séguin-Swartz 1992).

It is generally assumed in the literature that sirodesmins are important in blackleg pathogenesis, in spite of the fact that synthesis of these compounds by *L. maculans* in the host tissue has not been fully substantiated (Pedras & Séguin-Swartz 1992). Although toxins and culture filtrates cause necrosis on plant tissues to which they are applied, one cannot be certain that sirodesmins play a role in blackleg pathogenesis, nor can a determination be made as to what exactly that role may be.

Some of the symptom development and specificity not attributable to phytotoxins may be due to cell-wall degrading enzymes. Polygalacturonase is the predominant pectic enzyme produced in culture, and both polygalacturonase and pectate lyase are produced in the host tissue, as well as carboxymethyl cellulase and *a*-arabinosidase (Easton & Rossall, 1985). After testing several strain-cultivar combinations, Easton & Rossall (1985) found the highest polygalacturonase and *a*-arabinosidase activities in the most susceptible host-pathogen interactions, while resistant interactions were associated with low, slowly accumulating levels of these enzymes.

Polygalacturonase in particular may play an important role in pathogenesis since levels of this enzyme peak before macroscopic disease symptoms become visible (Easton & Rossall 1985). Fungal enzymes play a role in the pathogenesis of the disease, and part of the infective ability of the aggressive isolates is attributable to enzyme production.

Role of cell surface carbohydrates in plant-microbe interactions

Cell surface contact and adhesion are prerequisites for the development of pathogenic or symbiotic relationships between microbes and host plants. *Verticillium albo-atrum* produces cellulase only when in direct contact with cellulose (Gupta & Heale 1970). Mendgen (1978) found that an FITC-labeled cell wall preparation of the bean rust pathogen *Uromyces phaseoli* adhered to host tissues and only poorly to non-host tissues. Using seven host-specific strains of *Ceratocysistis fimbriata* and extracts from different hosts, Kojima & Uritani (1974) agglutinated spores of non-compatible strains and failed to agglutinate spores of compatible strains. Similar results were obtained by Chaboud & Lalonde (1983) who found that host-specific isolates of the actinorrhizal symbiont *Frankia* differentially bound to FITC-labeled lectins with different carbohydrate-binding specificities. Studies using lectins have successfully measured variability between *Rhizobium* species (Bohlool & Schmidt, 1974) and between two *formae speciales* of *Fusarium oxysporum* (Boyer & Charest, 1989).

Differential reactions between aggressive and non-aggressive *L. maculans* isolates may be correlated to variability among these strains with respect to cell-surface carbohydrates. If this hypothesis is correct, blackleg resistance of particular *B. napus* lines may be at least partially explained by host recognition of some *L. maculans* strains or pathotypes. That is, some incompatible (resistant) host pathogen interactions may be conditioned by host recognition of pathotype-specific *L. maculans* surface carbohydrates. If this is the case, isolates of *L. maculans* differing in their ability to infect particular *B. napus* lines or cultivars should also differ in cell-surface carbohydrate composition.

Host defense responses to infection

Brassica napus plants respond in a number of ways to infection by *L. maculans*. Moreover, these defense reactions vary in form and extent according to which *L. maculans* strain is responsible for infection. A series of important ultrastructural studies of the infection pathway of the blackleg fungus were made by K. E. Hammond and B. E. Lewis. They found that limitation of lesions was associated with lignin and callose deposition, as well as induced cambium formation (Hammond & Lewis 1987a). These changes occur in advance of the hyphal front, raising the possibility of the existence of a low-molecular weight chemical moiety which acts as a trigger of defense responses. An additional host response is accumulation of calcium in, but not outside of lesions (Hammond & Lewis 1986). Elevated levels of calcium were associated with the inner surfaces of cell walls which had accumulated electron-dense lignin-like material. That calcium plays a role in *L. maculans* pathogenesis is apparent by significant reductions in lesion size on *B. napus* stems infiltrated with $CaCl_2$ (Hammond & Lewis 1986).

Elicitation of phytoalexins in *B. napus* constitutes a second form of host defense response to infection by *L. maculans*. Phytoalexins are non-specific antimicrobial compounds that are synthesized *de novo* and accumulated by plants in response to virus, bacterial, fungal, nematode or insect attack; synthesis and accumulation may also be elicited by abiotic agents, such as heavy metallic salts, ultraviolet radiation or wounding (Bailey 1982, Mansfield 1982, Paxton 1981). To date, thirteen phytoalexins have been characterized in Brassicaceae (Conn 1991), three of which, cyclobrassinin (Dahiya & Rimmer 1988, Devys et al. 1990, Peterka & Schlösser 1990), methoxybrassinin (Dahiya & Rimmer 1988, Rouxel et al. 1991) and spirobrassinin (Pedras & Séguin-Swartz 1992) have been identified in *B. napus* in response to *L. maculans* infection.

Elicitation and accumulation of these phytoalexins is associated with specificity of *B. napus-L. maculans* interactions. Dahiya & Rimmer (1989) found that a weakly virulent isolate elicited production of methoxybrassinin and cyclobrassinin, whereas an aggressive isolate elicited cyclobrassinin production only. Moreover, the quantity of phytoalexin produced was greater in response to infection of *B. juncea* cv. Cutlass by the weakly virulent isolate (Dahiya & Rimmer 1989). Dahiya & Rimmer (1989) report in the same paper that resistant lines of *B. napus*, *B. juncea* and *B. rapa* accumulate larger quantities of phytoalexins than susceptible lines when challenged with a non-aggressive *L. maculans* isolate. In contrast, Peterka & Schlösser (1990) were unable to find a correlation between disease severity and phytoalexin accumulation in cotyledons. This suggests that phytoalexin induction may be a defense response resticted to true leaves. Resistance in cotyledons may be based on a different mechanism. Interestingly, Peterka & Schlösser (1990) observed that a non-aggressive isolate of *L. maculans* induced chitinase activity in cotyledons, whereas an aggressive isolate was able to suppress induction.

Host range

Cruciferous hosts are attacked by *L. maculans*, and susceptibility varies among members of the family. According to Dixon (1981), highly susceptible species include cabbage (*B. oleracea* var. *capitata*), Chinese cabbage (*B. pekinensis*) and *B. chinensis*) Brussels sprouts (*B. oleracea* var. *gemmifera*), some swede varieties (*B. napus* var. *napobrassica*), white mustard (*B. hirta*) and kohlrabi (*B. caulorapa*). Hosts of moderate susceptibility include broccoli (*B. oleracea* var. *italica*), cauliflower (*B.oleracea* var. *botrytis*), rape (*B. napus* var. *oleifera* and *B. rapa*), collard and kale (*B.oleracea* var. *acephala*), wild radish (*Raphanus raphanistrum*) and black mustard (*B. nigra*). Moderately- to highly-resistant species include some turnip and swede varieties, Indian mustard (*B. juncea*) and garden cress (*Lepidium sativum*) (Dixon 1981). Very resistant hosts are horseradish (*A. rusticana*), stinkweed (*Thlaspi arvense*), yellow rocket (*Barbarea vulgaris*), shepherd's purse (*Capsella bursa-pastoris*), ball mustard (*Neslia paniculata*) and pepper cress (*L. densiflorum*). Note that some hosts are weedy species. Specialized strains specifically virulent towards *Descurania* sp., *Sisymbrium* sp. and *T. arvense* exist in western Canada, but these strains have not been shown to be aggressive towards oilseed *B. napus* (Petrie 1969, Williams 1992). *Leptosphaeria. maculans* has also been isolated from oilseed radish (*Raphanus sativus*) (Petrie & Vanterpool 1974).

Epidemiology

Development of blackleg symptoms is favored by high humidity and higher temperatures. Wood & Barbetti (1977a) found that extended periods of 100% relative humidity increased the incidence of diseased plants, while disease incidence was lower when either relative humidity was decreased, or when surface moisture was absent. McGee (1977) and Xi et al. (1990) found that disease incidence increased under field conditions when more moisture was available. It is not clear from the literature, however, whether moisture conditions affect symptom development directly, or indirectly via spore germination and infection.

Development of leaf and crown lesions is slowed by low temperatures, particularly those less than 10° C (Alabouvette et al. 1974, Lacoste et al. 1969, McGee 1977), although lesion development on cotyledons slows as temperatures increase from 8 to 15° C (McGee 1977). The optimum temperature for canker development is within the 20-24° C range (Barbetti 1975, Brunin & Lacoste 1970, Gladders & Musa 1980, Helms & Cruikshank 1979, McGee & Petrie 1979, Ndimande 1976). Leaf and stem canker morphology is also affected by temperature, as systemic leaf infections and formation of large stem cankers are more common between 15 and 25° C, whereas local infections and small non-sporulating cankers occur more readily at lower temperatures (Barbetti 1975, Hammond & Lewis 1987b). Blackleg symptoms do not develop at all at high temperatures (> 28° C) (Barbetti 1975, Brunin & Lacoste 1970, Gladders & Musa 1980, Helms & Cruikshank 1979, McGee & Petrie 1979, Ndimande 1976).

The rate of ascospore release is greatest between temperatures of 8° - 12° C (McGee 1977). The spores are wind-dispersed, the largest number of ascospores being caught in spore traps at low wind speeds (Krüger & Wittern 1985). High humidity, dew or rain are required for ascospore release (Krüger & Wittern 1985, McGee 1977). The effective range of wind dispersal of ascospores is somewhat limited, as disease incidence decreases rapidly 2-5 km from infected fields (Gladders & Musa 1980, Petrie 1978).

B. napus becomes less susceptible after the second leaf stage, and wounding is generally a prerequisite for infection during the four to six leaf stage (Brunin & Lacoste 1970, Ndimande 1976, Xi et al. 1991). McGee & Petrie (1979) were able to induce severe disease symptoms only in plants inoculated in the crown before the six leaf stage. McGee & Emmett (1977) found that infections causing serious cankering are initiated before flowering. It is therefore apparent that *B. napus* plants become more resistant to the pathogen with advancing maturity.

Virulent strains of *L. maculans* have adapted to increasing host resistance by discharging ascospores during periods of high host susceptibility. Moreover, timing of ascospore discharge varies with geographical location since environmental conditions vary from one area to the next. In France, the period of highest ascospore discharge rate corresponds with the first two months of growth of winter rapeseed (Alabouvette & Brunin 1970). Similarly, in Sweden, formation of the telomorphic stage (August to September) corresponds to the most susceptible stage of the crop (Ndimande 1976). Ascospore discharge also corresponds to the most susceptible crop stage in Canada (McGee & Petrie 1979) and Australia (McGee 1977). In Australia, disease severity can be reduced by sowing crops in August, a period of decreased ascospore discharge (McGee 1977).

Aggressive and non-aggressive strains discharge ascospores at different times. McGee & Petrie (1979) found that strains of low virulence do not release ascospores until July, by which time rape plants can no longer be considered to be fully susceptible, whereas aggressive strains release ascospores in May, allowing severe infections to occur. Rempel & Hall (1992) found that an overwintering period is not required for maturation of pseudothecia or ascospore release, therby allowing infection of winter rape via ascospores produced on the current year's spring canola crop residues.

Pycnidiospores are the most important form of secondary inoculum, providing a mechanism whereby the disease can be spread even when ascospores are lacking (Barbetti 1976, Ndimande 1976). Dispersal is primarily by rain-splash, allowing inoculum to spread several metres from an infected plant (Barbetti 1976, Petrie 1978).

Wounding may be an important prerequisite for infection. Infection is more reliable under greenhouse conditions when plants are wounded prior to inoculation (Hammond et al. 1985, Ndimande 1976). This may explain why herbicide injury (Petrie 1973) and insect damage increase disease severity (Newman 1984a).

Infected crop residues play an important role in the epidemiology of blackleg. The pathogen is able to overwinter in lignified canola stems, which serve as an inoculum pool the following spring. When infected residues are present, disease severity varies with the severity of the disease in the crop producing the residue (McGee 1977) and with the longevity of the residue itself. Mycelium, pseudothecia and ascospores, as well as pycnidia and conidia have been found in *B. napus* residues from one to five years after initial infection (Alabouvette & Brunin 1970, Lacoste et al. 1969, McGee 1977). In

Canada, infected three-year old residue has been associated with severe yield losses (Petrie 1986b). Similar conclusions have been made with respect to Western Australia (Bokor et al. 1975), and Sweden (Ndimande 1976), where infected residues have been responsible for high disease severities three to five and two years after initial infection, respectively. Alabouvette & Brunin (1970) speculated that the fungus survives in the soil as long as lignified residues remain, raising the possibility that the crops could become infected from residues brought to the surface by cultivation.

Leptosphaeria maculans cannot normally survive in soil in the absence of crop residues (Gabrielson 1983), although there is one report of the fungus behaving as an entirely subterranean pathogen causing root rot of Brussels sprouts (Snyder & Baker 1950).

Infected seed plays a very important role in the epidemiology of blackleg. The seed infection phase of the disease is thought to be important in introducing the pathogen to *B. napus* and *B. rapa* crops in geographical areas not previously affected by the disease (Gabrielson 1983, Hall 1992, Petrie & Vanterpool 1974). Furthermore, *L. maculans* can survive for 2 to 10 years on or in crucifer seed (Gabrielson 1983).

Various pathways for the infection of seeds have been proposed. These range from systemic infection (Wood & Barbetti 1977a, Gabrielson 1983) to localized infection through the silique wall (Henderson 1918, Hall 1992, Bonman & Gabrielson 1981) to infection via the stigmatic end of the siliques after inoculum transmission by insects (Petrie & Vanterpool 1974).

Seed infection does not, however, play an important role in secondary spread or in local dispersal once the disease is endemic to an area. Usually only a small percentage of seeds within a sample are infected, even when samples are taken from areas in which the disease is has been long established or when a large percentage of fields are found to be contaminated (Chigogora & Hall 1990; Hall 1992; Humpherson-Jones 1984, 1985; McGee 1977; Petrie 1979; Petrie & Vanterpool 1974). Moreover, the frequency of infected seed does not correlate well with disease incidence in areas in which the disease is established (McGee 1977), although positive correlations are found when the pathogen is introduced into previously uncontaminated areas (Wood & Barbetti 1977b).

Non-rape species, including *Sinapsis arvensis* (Petrie 1979), *B. carinata*, *B. nigra*, *Raphanus sativus*, *S. alba* and *T. arvense*. as well as volunteer *B*.

napus and *B. rapa* may be infected and serve as inoculum pools (Gugel et al. 1990). Vegetable crucifers, including *B. oleracea*, *B. napus* and *B. rapa*, may also be susceptible to those *L. maculans* isolates virulent on oleiferous *B. napus* (Humpherson-Jones 1983a; 1986), although the literature is not clear on this point (Delwiche & Williams 1979, Hall 1992).

Cultural and chemical control

The discovery of the importance of crop residues and seed-borne inoculum in the epidemiology of blackleg disease of canola have led to a number of disease control recommendations. Many of these recommendations are designed to reduce inoculum levels.

Crop rotation is an effective method of reducing inoculum levels since this practice allows decomposition of crop residues, thereby reducing the numbers of ascospores and pycnidiospores from pseudothecia and overwintered pycnidia. In western Canada the current recommendation is for three years between successive canola crops (Gugel & Petrie 1992, Thomas 1984, Petrie 1986b).

Deep burial of residues effectively prevents infection of *B. napus* (Alabouvette & Brunin 1970, Evans 1988, Gladders & Musa 1980, Krüger & Wittern 1985, Thomas 1984). Moreover, this practice may increase the rate of natural decomposition of residues, although decomposition rates are dependent on available moisture (Gugel & Petrie 1992). Care must be taken during cultivation to avoid reintroduction of partially-decomposed residues to the soil surface.

Control of volunteer rapeseed and weed hosts of *L. maculans* is another control strategy designed to reduce inoculum quantity. This practice is particularly important when *B. napus* is rotated with non-cruciferous crops, since volunteer plants and cruciferous weeds may harbor the pathogen (Evans 1988, Thomas 1984).

The distribution of infected *Brassica* seed is restricted to prevent the introduction of the pathogen into unaffected regions, such as the Peace River region in the province of Alberta (Harrison 1988, 1989, 1990; Harrison & Loland 1991). Field inspection (Gugel & Petrie 1992), seed testing (Kharbanda 1989) and seed certification (Gugel & Petrie 1992) programs have been instituted. Furthermore, regulations and legal penalties have been enacted concerning the virulent strain of *L. maculans* (Province of Alberta 1984).

Chemical measures have also been considered for the control of blackleg. Kharbanda (1992) found that iprodione and prochloraz applied as foliar sprays controlled the disease in growth chamber tests; the latter compound also provided effective control measure in field tests with artificial inoculation. Prochloraz failed to protect plants, however, under natural inoculum and heavy disease pressure, even when two fungicide treatments were applied. To date, no foliar spray for control of blackleg is registered.

Fungicide treatment of seed has been shown to be more effective (Evans 1988, Gugel & Petrie 1992, Humpherson-Jones 1986, Thomas 1984, Wood & Barbetti 1977b). Application of flutriafol to canola as a coating on superphosphate fertilizer has significantly reduced yield losses to blackleg in Australia (Ballinger et al. 1988a, 1988b) although this method did not effectively control the disease in western Canada (Xi et al. 1991). The aim of seed treatment is to prevent introduction of *L. maculans* into unaffected regions, since seed-borne inoculum in not important once the disease is established in an area (McGee 1977).

Resistance

A plant is said to be resistant to disease when the progress of the pathogen through host tissue is partially or completely limited by pre-formed or induced host defense responses (Parlevliet 1989, Wang & van der Kamp 1992). Tolerance, on the other hand, means that yield loss to disease is limited, even though infection and colonization of the host may not be restricted (Parlevleit 1989, Wang & van der Kamp 1992). Thus, a plant may be both susceptible and tolerant to the same disease.

A large emphasis has been placed on the incorporation of blackleg resistance into *B. napus*, since seed treatment is not an effective disease control in areas to which *L. maculans* is endemic, and since effective foliar fungicide treatments are unavailable. Resistance to blackleg has been found in *B. napus* (aacc), *B. oleracea* (cc) and *B. insularis* (cc), all of which have c genome chromosomes. Good levels of blackleg resistance under Canadian conditions have been found in summer and winter *B. napus* cultivars originating in Australia, Japan and Europe (Rimmer & van den Berg 1992).

Although rare, some resistance to *L. maculans* has been found in some cabbage accessions (*B. oleracea*) (Monteiro & Williams 1989). *B. insularis*, a wild member of the *B. oleracea* group, possesses a hypersensitive type of resistance (Mithen et al. 1987).

Distinctions are usually made between resistance in *B. napus* at the cotyledon and adult plant stages. Some workers obtained good correlations between cotyledon screens in controlled environments and resistance in the field (Cargeeg & Thurling 1980, Stringam & Bansal, unpublished data), while others obtained poor correlations (Newman & Bailey 1987, Wittern & Krüger 1985). The winter-type *B. napus* cultivar Jet Neuf is susceptible to some *L. maculans* isolates at the cotyledon stage and is tolerant at the adult stage (Badawy et al. 1991). The extent to which cotyledon screening results correspond to field resistance appears to vary with the *Brassica* species under evaluation, the accession being evaluated, the type and concentration of the inoculum used, and the inoculation and evaluation procedures employed. Consequently, it is not clear whether resistance in these two stages is controlled by separate genes.

Several models of the inheritance of blackleg resistance in *B. napus* have been proposed. These vary from monogenic dominant (Stringam et al. 1992) to two-gene dominant additive (Sawatzky 1989) to polygenic systems (Cargeeg & Thurling 1980) in the adult stage. Seedling resistance is thought to be conditioned by a single recessive gene (Sawatzky 1989), a single dominant gene (Stringam et al. 1992), or one of two linked dominant genes (Delwiche 1980). Judging from the range of models proposed in the literature, it appears that several types of resistance are available in *B. napus*.

An important source of resistance to *L. maculans* among the cultivated *Brassica species* is the hypersensitive-type resistance associated with the b genome. According to Rimmer & van den Berg (1992), this type of resistance is conditioned by two nuclear genes with dominant recessive epistatic action. Species exhibiting this type of resistance include *B. carinata*, *B. nigra* and *B. juncea*. (Sjödén & Glimelius 1988), although some of the resistance carried by *B. carinata* may be located in the c genome, since crosses of *B. napus* with *B. carinata* behave differently than crosses of *B. napus* with *B. juncea* (Sacristian & Gerdemann 1986). The *B. juncea* type of resistance to *L. maculans* is important from an agronomic standpoint because of the possibility of interspecific transfer of resistance to other Brassicas, and because of the potential of *B. juncea* as a canola oil crop (Woods et al. 1991).

Some *B. juncea* accessions are susceptible to *L. maculans*. These lines will show no symptoms on aerial plant parts, but rather the roots are discolored, with macroscopic symptoms identical to those of root rot if plants are infected at an early stage (Keri 1991).

Good levels of blackleg resistance have been incorporated into oleiferous *B. napus*, even though the exact nature of inheritance of the trait has not been fully elucidated. An illustration of this is the high level of disease control achieved in Europe since the introduction of *B. napus* var. *biennis* cv. Jet Neuf, which has served as a source of resistance in many subsequent European cultivars (Rimmer & van den Berg 1992).

Pathogen variability for virulence

Variability in pathogenic adaptation reflects the type and number of virulence-conferring genes a particular pathogen possesses. These range from the few major, host-specific genes carried by specialized pathogens to the many minor, non-specific virulence genes of broad pathogens. The virulence gene complement of a pathogen affects the choice of strategies for introgressing resistance into new cultivars.

Some species of phytopathogenic fungi are divisible into physiological races defined on the basis of reaction on a set of differential cultivars. These fungi are typically biotrophic, obligate parasites with a limited host range. The flax rust fungus, *Melampsora lini*, is the classic example of this type of pathogen (Flor 1955). Host-pathogen interactions of *M. lini* and its sole host (*Linum usitatissimum*) occur on a gene-for-gene basis, where incompatibility (resistance/avirulence) results only from specific combination of resistance and avirulence genes (Flor 1946, 1955; Johnson & Knott 1992; Kerr 1987). Resistance and virulence in gene-for-gene systems are simply inherited, with each virulence-resistance gene pair having a major, non-additive effect. Variation for virulence among fungal genotypes of these pathogens is generally discontinuous or qualitative, with races falling into fully virulent or fully avirulent categories with respect to any particular differential host. Intermediate interaction phenotypes are absent or few in number. Disease resistance can be lost through race change at a single virulence locus.

Although new resistances may be incorporated with the same techniques used to introgress any other desirable trait (Allard 1960), consideration must be given to the fact that serial incorporation of new resistance genes may be required over time to replace resistances negated by pathogen race change. Strategies such as resistance gene pyramiding (Mundt 1990) or multiline varieties may be used to slow the loss of resistance.

The second extreme of phytopathogenic adaptation is represented by polyphagous, pathogenically unspecialized fungi such as *Sclerotinia sclerotiorum*. Variability for virulence of these pathogens is quantitative and unspecialized, and specific interaction of host and pathogen genotypes at the cultivar level is absent or difficult to discern. This is because resistance and virulence genes, which do not interact on a gene-for-gene basis, have small, additive individual effects (Parlevliet 1989). Breeding for resistance to these pathogens involves selection for small, incremental increases in resistance. Often a compromise between increased resistance and linked undesirable agronomic traits must often be made, since resistance genes often affect fundamental plant processes (Bruehl 1983). For example, resistance to root rot in barley caused by *Pythium arrhenomyces* is associated with varietal adaptation to photoperiod (Bruehl 1955). Inheritance of photoperiod response in barley is complex, as is resistance to Pythium root rot (Bruehl 1983). Consequently, any selection for root rot resistance may also involve selection for undesirable photoperiod responses. Despite these problems, multigenic resistance to broad pathogens is sought after since it is considered permanent due to its immunity to pathogen race change (Parlevliet 1989).

It is uncertain where *L. maculans* falls in the range of pathogenic adaptation. It is a necrotrophic fungus, but the infection path is partially biotrophic (Hammond & Lewis 1986, Nathaniels & Taylor 1983). It attacks several species within Brassicaceae, yet specificity at the cultivar level is detectable.

Differential reactions among *B. napus* cultivars have been obtained by a number of workers with respect to sets of virulent *L. maculans* isolates. The results of these studies are variable. Newman (1984b) placed English isolates into two groups based on stem reactions of the cultivars Garant, Norli or Quinta when systemically infected by the pathogen. Working with aggressive Australian isolates, Cargeeg & Thurling (1980) found significant cultivar × isolate interactions after systemic infection of the cultivars Ceska, Chisaya and

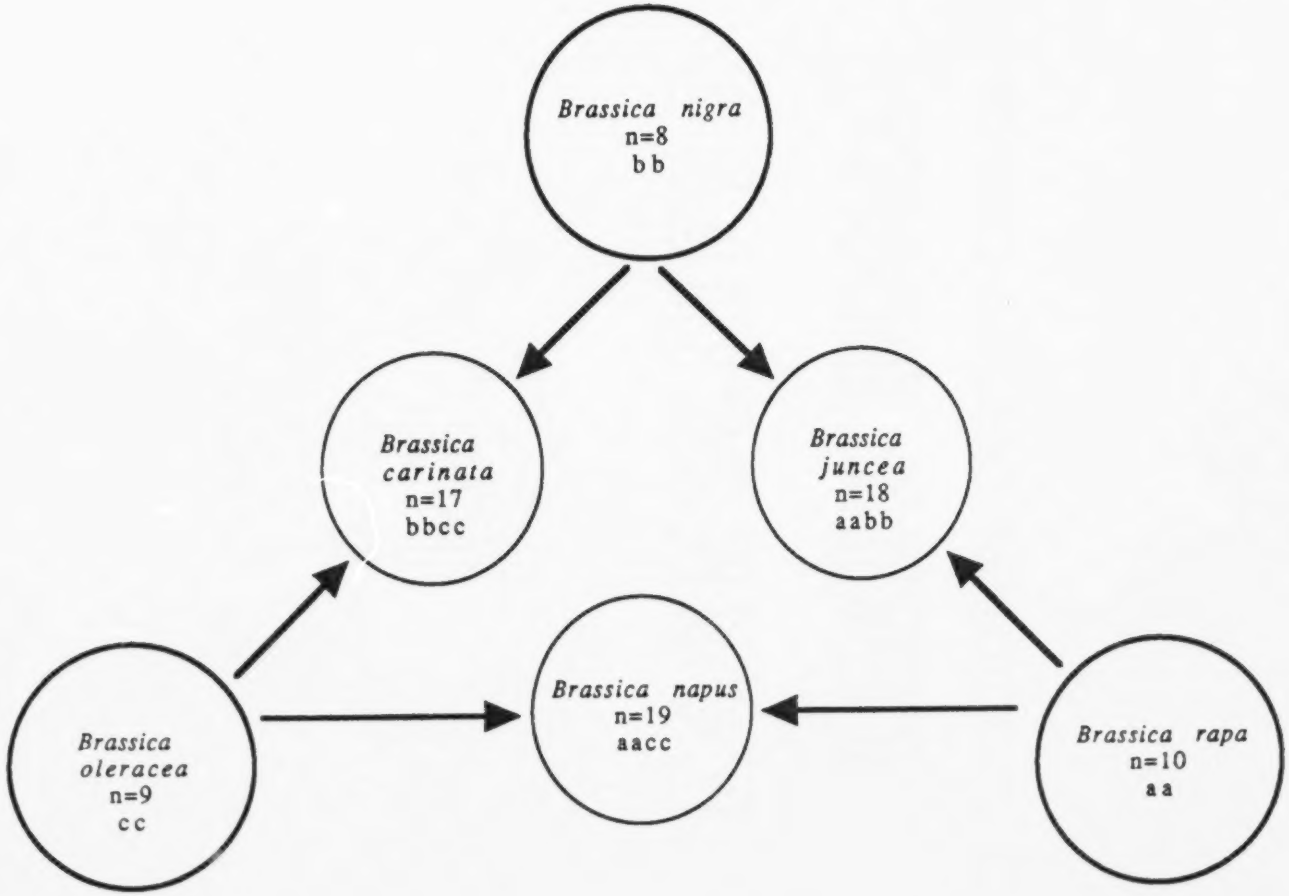
Wesreo. Koch et al. (1991) have developed a set of four differential *B. napus* cultivars which allow classification of *L. maculans* isolates into one of four pathogenicity groups (PG1-PG4). Weakly virulent strains fall into PG1, based on the reaction they produce when inoculated onto cotyledons of the susceptible cultivar Westar. Aggressive isolates are divisible into three groups (PG2-PG4) on the basis of their reaction on the cultivars Glacier and Quinta. Western Canadian *L. maculans* isolates appear to belong to PG2 only (Mengistu et al. 1991, Rimmer & van den Berg 1992), although it is unclear whether western Canadian isolates of *L. maculans* differ with respect to their complement of virulence genes, since these isolates react differentially when challenging certain *B. napus* and *B. juncea* accessions (Kutcher 1990).

Rimmer & van den Berg (1992) hypothesize that two genes conditioning avirulence exist within *L. maculans*, and that these genes interact with host resistance genes in a manner predicted by the gene-for-gene hypothesis. These results, however, apply to the segregation of virulence genes in the progeny of a single cross, in relation to a single susceptible cultivar. Clearly, the study of inheritance of virulence in *L. maculans* is still in its early stages.

Objectives of the Thesis

The objectives of this thesis were to study the variability in aggressivity of western Canadian isolates of *L. maculans* with respect to a set of differential cultivars, and also to evaluate selected morphological characteristics and *in vitro* pigment production of these isolates. A second objective was to associate the variability among isolates with respect to the above characteristics with variation in cell-surface carbohydrates, based on the hypothesis that these moieties are involved in host-pathogen recognition.

Figure 1.1. Cytogenetic interrelationships among oleiferous *Brassica* species. Arrows indicate genome contributions of monogenomic progenetor species (dark cicles) to allotetraploids (light circles).


Brassica nigra
n=8
b b
Brassica carinata
n=17
bbcc
Brassica juncea
n=18
aabb
Brassica oleracea
n=9
c c
Brassica napus
n=19
aacc
Brassica rapa
n=10
a a

Adapted from U (1935)

Figure 1.2. Stem canker caused by *Leptosphaeria maculans* on stem of *Brassica napus*. Note pycnidia within the central area of the canker. Also note cracked stem (arrow). (Approximately 5.7 ×).

Figure 1.3. Pseudothecia of *Leptosphaeria maculans* on a three year old stem of *Brassica napus*. Pseudothecia are interspersed with pycnidia of the anamorph (*Phoma lingam*). (Approximately 6.5 ×).

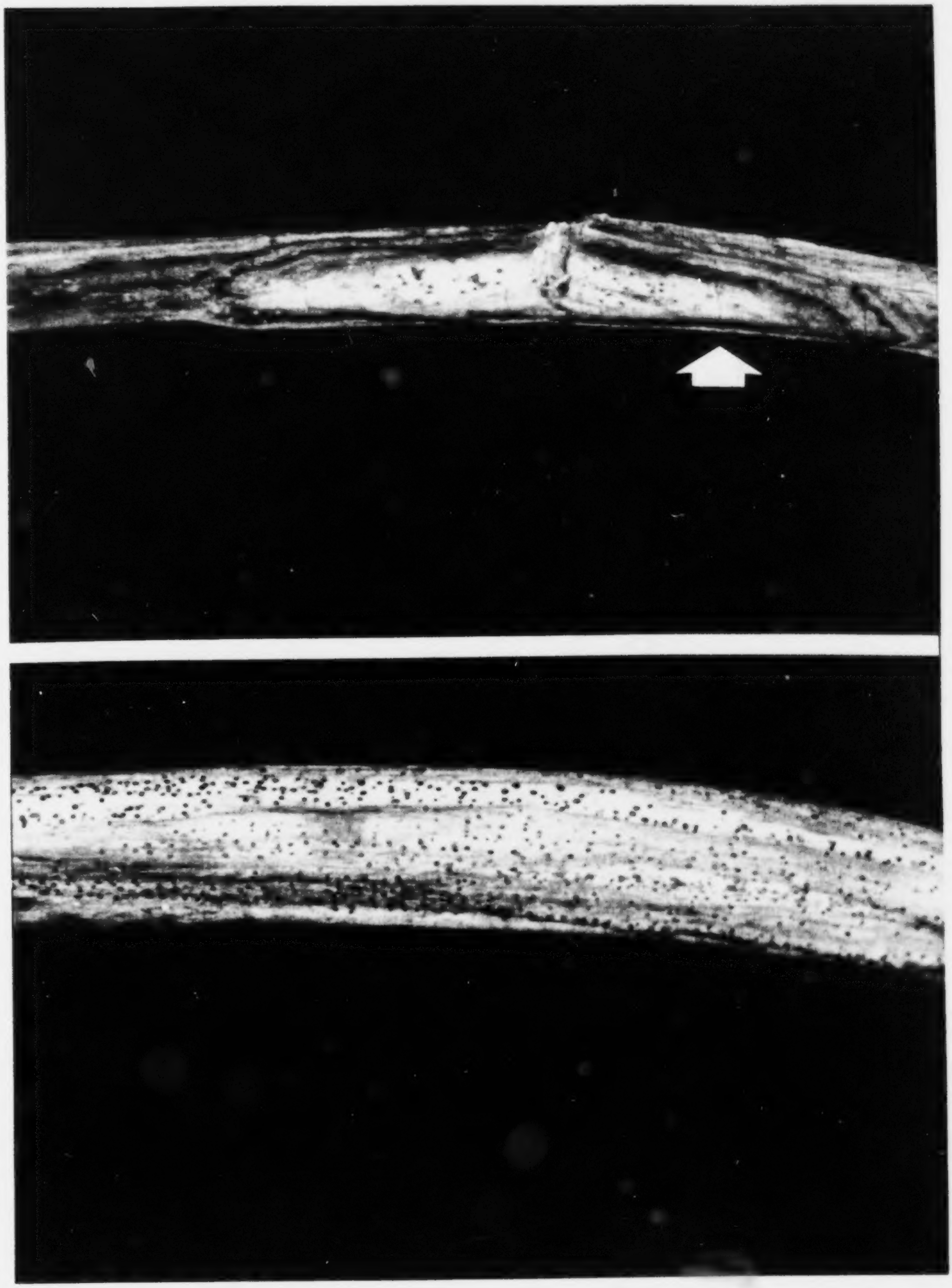
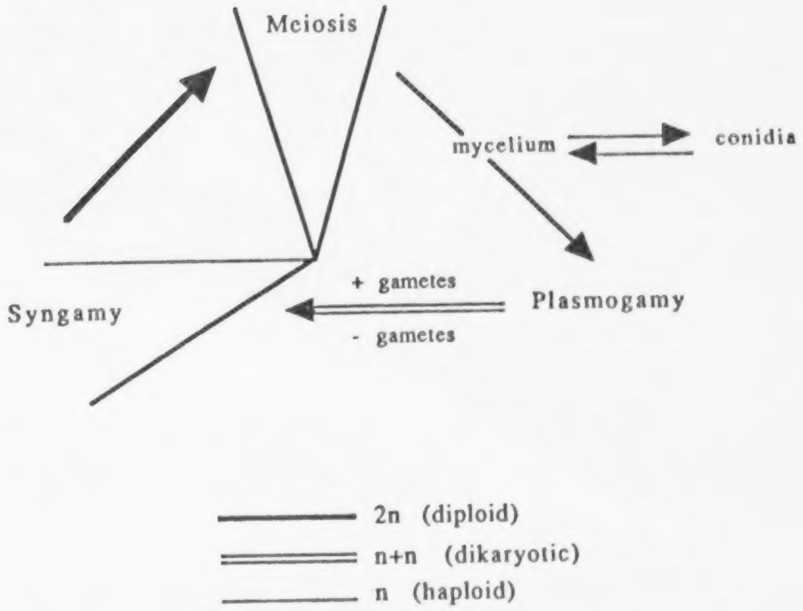


Figure 1.4. Generalized ascomycete lifecycle. Note that the haplont (n) is conspicuous, whereas the diplont (2n) is composed of a few pre-meiotic cells.


Meiosis
mycelium
conidia
Plasmogamy
+ gametes
- gametes
Syngamy
2n (diploid)
n+n (dikaryotic)
n (haploid)

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Chapter 2

Characterization of Diversity in Pathogenicity Profiles and Cultural Characteristics of Western Canadian Isolates of *Leptosphaeria maculans*.

Introduction

Blackleg, caused by *Leptosphaeria maculans* (Desmaz.) Ces. et de Not. (anamorph *Phoma lingam* (Tode ex Fr.) Desm.) is one of the most important diseases of canola (*Brassica rapa* L. (syn. *B. campestris* L.) and *B. napus* L.). Yield losses can exceed 50% (Thomas 1984). Isolates of *L. maculans* attacking *B. napus* are broadly divisible into weakly virulent (non-aggressive) and virulent (aggressive) strains. Weakly virulent strains of the blackleg fungus have been present in western Canada at least since 1957 (Vanterpool 1959, 1962), and are not known to cause canola crop losses of any consequence. A strain of *L. maculans* capable of causing severe basal stem cankering was noted in Saskatchewan in 1976 (Petrie 1978). It was found that 10 and 17% of the fields surveyed in 1976 and 1977, respectively, were infected with the pathogen (Petrie 1978). The virulent *L. maculans* has since spread throughout the prairie provinces, to the extent that by 1991 61% (Van Den Berg et al. 1992) 76% (Kirkham 1992) and 50% (Evans et al. 1992) of fields surveyed in Manitoba, north-eastern Saskatchewan and Alberta, respectively, were affected by the fungus.

Variability in pathogenic adaptation is detectable among virulent isolates. Thurling and Venn (1977) found highly significant interactions between host cultivars and pathogen isolates, where the virulent *L. maculans* isolates used originated from three widely separated collection sites in Australia. Similarly, Bonman et al. (1981) found a range of virulence expressed within aggressive Australian isolates inoculated onto cabbage cotyledons and transplanted cabbage plants. Cargeeg and Thurling (1981) found geographic variation in frequency of specific virulence genes. Koch et al. (1991) were able to subdivide virulent strains of *L. maculans* into three subgroups on the basis of disease reaction of the cotyledons of the *B. napus* cvs. Westar, Quinta and Glacier. They also found that RFLP-based phylogenetic groups were associated with pathogenicity groups. Using the differential cultivar system developed by Koch and his coworkers (1991), Mengistu et al. (1991) found that Canadian isolates from Manitoba and Saskatchewan all fell into the same pathogenicity group. Variability in pathogenic specificity of *L. maculans* isolated from *B. napus* fields in Alberta has not been studied.

Certain cultural traits, including growth rates and colony morphology, have been associated with virulence in *L. maculans* (Koch et al. 1989, McGee & Petrie 1978). Furthermore, a brown pigment commonly seen when non-aggressive strains of the fungus are cultured is not observed when aggressive isolates are grown (Delwiche 1980, Humpherson-Jones 1983, Koch et al. 1989, McGee & Petrie 1978). Evaluation of differences in these morphological and cultural characteristics may reveal variability among isolates not discernable simply through virulence testing on differential cultivars.

The development of resistant *B. napus* cultivars appears to be the best strategy for the control of the disease since cultural and chemical controls are not fully effective (Gugel & Petrie 1992). Unfortunately, no fully resistant cultivars are currently available, although some accessions and cultivars possess intermediate resistance (Jesperson 1990) or high levels of tolerance (Stringam, G.R., personal communication). The effectiveness of blackleg resistance breeding programs would be greatly improved by a more complete documentation of diversity in pathogenicity profiles of *L. maculans* in relation to several *B. napus* genotypes.

The objectives of this study, therefore, were to study the variability in aggressivity of western Canadian isolates of *L. maculans* with respect to the differential cultivars Westar, Quinta and Glacier, and the Australian blackleg resistant cv. Maluka. The morphological characteristics and *in vitro* pigment production of these isolates were also evaluated.

Materials and methods

Plant Germplasm. Plant materials used are listed in Table 2.1. Seed of the *B. napus* cultivars Westar, Glacier and Maluka were obtained from the germplasm collection of the Department of Plant Science, University of Alberta. *Brassica napus* cv.Quinta seed was obtained from Norddeutsche Pflanzenzucht, Hans-Georg Lemke K.G. (Hohenlieth, Germany). All other seed was provided by Dr. Gary R. Stringam, Department of Plant Science, University of Alberta.

Seeds were sown in a soil-free growth medium in 32-celled plastic trays, two seedlings per cell. Seedlings were maintained in a greenhouse (21° ± 4° C, 16 h photoperiod supplemented with 400 W high pressure sodium lamps) for six or seven days prior to inoculation. Seedlings were grown in the greenhouse between the months of September through June.

Fungus culture. *Leptosphaeria maculans* cultures were grown on 2% (w/v) V-8 juice agar (V8A) (200 ml L^{-1}V-8 juice, 50 mg L^{-1} rose bengal and 3 g L^{-1} $CaCO_3$) in 100 × 15 mm Petrie plates. Plates were incubated at ambient temperature (ca. 23° C) under cool white florescent tubes (ca. 170 $\mu E\ m^{-2}\ sec^{-1}$) for a 16 hour photoperiod.

Isolates. Infected canola stems (*B. napus* and *B. rapa*) were collected in Manitoba, Saskatchewan and Alberta from September to November, 1989 (Table 2.2). *Leptosphaeria maculans* was isolated as the conidial state by placing stem sections bearing pycnidia into moist chambers for 24 to 48 hours. Exuded pycnidiospores were transferred to V8A. Plates to which pycnidiospores were initially transferred were amended with 100 mg L^{-1} streptomycin sulfate after autoclaving to inhibit bacterial growth. Isolates were subcultured until pure cultures were obtained.

Single spore isolates were obtained from pure cultures by transferring a small amount of pycnidial exudate to 10 ml of distilled sterile water, agitating the test tube for a few seconds in a vortex mixer and streaking a few drops of the resulting mixture onto 2% water agar (WA). Inoculated WA plates were incubated at ambient temperatures (ca. 23° C) for 24 h. Single germinated conidia were transferred to V8A with a sterile 25 gauge hypodermic needle. Some single spore isolates were obtained directly from host tissue by placing small stem pieces bearing pycnidia into 10 ml sterile water. Pieces were left in water for approximately 30 minutes to ensure that conidia were exuded. A few drops of the resulting spore suspension were drawn off with a sterile Pasteur pipette and spread on WA. Single germinated conidia were then transferred to V8A in the usual manner.

Cryogenic storage. Sporulating single-spore cultures of the conidial state of *L. maculans* were flooded with approximately 20 ml 20% glycerol (v/v) used as a cryoprotectant. Cultures were then rubbed gently with a glass rod to dislodge the conidia. The conidial suspension was then aseptically transferred to labelled sterile polypropylene cryogenic vials in aliquots of roughly 1 ml using a Pasteur pipette. Several vials were prepared from each isolate. The vials were cooled overnight at 4° C, then suspended in liquid nitrogen vapor for 24 h to approximately -184° C using a Handi Freezer Tray (Union Carbide; Model P/N R036-8C15). Vapor-cooled vials were then immersed in liquid nitrogen, attached to labelled metal holders and transferred to a Union Carbide 35HVC Cryogenic Refrigerator filled with liquid nitrogen for storage at -196° C until required.

Cultures were revived by placing the cryogenic vials containing suspended conidiospores into water (35 to 45° C) until thawed. The suspensions were allowed to return to room temperature, and then transferred directly to V8A. The glycerol cryoprotectant solution did not appear to affect fungal growth. Revived cultures were subcultured at least once before use.

Isolate virulence screens. Virulence profiles of *L. maculans* were determined by inoculating cotyledons of the *B. napus* cultivars, Westar, Quinta, Glacier and Maluka, with the isolates listed in Table 2.2.

The inoculum was prepared by transferring 2 to 5 exuding pycnidia to 5×10 mm test tubes containing sterile distilled water and allowing 15-30 minutes for dispersal of conidia. Pycnidia were then removed from the water and the spore suspensions were adjusted to 1.0×10^6 conidia ml^{-1} using an American Optical Brightline Hemacytometer (American Optical Corporation, Buffalo NY; Cat. No. 1492).

The cotyledons of six- or seven-day old plants were inoculated by piercing each cotyledon with a no. 1 insect pin and placing a 10 μl inoculum droplet over the wound. Plants were incubated for 40 to 48 h at high humidity by placing covered trays into a plastic-covered chamber equipped with ultrasonic humidifiers (Figure 2.1). Plants were subsequently removed from the humid chamber and grown for a further 8 days under the normal culture conditions described above.

Four replicate pots containing eight seedlings were evaluated for each isolate × cultivar combination. Pots were arranged into a completely randomized block design. Symptoms (Figure 2.2) were evaluated according a 0 (resistant) to 9 (susceptible) Interaction Phenotype (IP) scale adapted from that of Williams (1985) (Table 2.3). Interaction phenotypes of 0 to 3 are indicative of host resistance, a rating of 5 is considered intermediate, and ratings of 7 and 9 indicate host susceptibility. Data consisted of the IP values of the most severely affected cotyledon of each seedling.

Disease severity (DS) values were calculated for each isolate × cultivar combination according to the formula used by Xi et al. (1990):

$$(1)\ \mathrm{DS} = \frac{\sum_{i=1}^{i} i\ \mathrm{f}j}{\mathrm{I} \sum_{i=1}^{i} \mathrm{f}j}\quad (100)$$

where i and $\mathrm{f}j$ are the IP values and the frequency of individual plants in the jth IP category, respectively, and I is the maximum category value (9 in this case).

Virulence screen confirmation experiment. An experiment was conducted to examine the reproducibility of the isolate screening data. Three randomly chosen weakly virulent *L. maculans* isolates (LMM89-1, LMS89-26 and BL88-22(4)), three randomly chosen virulent isolates (LMM89-3, LMA89-64 and LMA90-3356), and the isolate originally collected in Germany (LMG90-112) were used to challenge seedlings of the same *B. napus* cultivars used in the original screening trials. Leroy, an isolate from Saskatchewan known to belong to the virulent strain of *L. maculans*, (Gugel et al. 1990) was included as a control.

Experimental design, plant and fungus culture, inoculation procedures and evaluation methodologies were identical to those used in the initial isolate virulence screening study. Disease severity values (Equation 1) fell outside of the 30-70% range and were, therefore, transformed using the arcsine (angular) transformation (Steel & Torrie 1980). Untransformed values are presented in all tables. Transformed data were then submitted to SAS (SAS Institute Inc., Cary, NC) for analysis of variance (ANOVA). In addition, transformed values were compared with corresponding data from the initial virulence screens through the application of the Wilcoxon signed rank test (Steel & Torrie 1980). This distribution-free method is a useful indicator of whether a change in a variable has occured, and is more powerful than a sign test since the magnitudes of the differences between paired samples is taken into account (Steel & Torrie 1980).

Confirmation of virulence towards *B. napus* cv. Maluka cotyledons. Seven aggressive isolates found to be virulent towards Maluka in the initial cotyledon screening tests were inoculated onto cotyledons of Westar, Maluka and Shiralee. 'Leroy' was included as a control since a mean disease severity of less than 50% was recorded for this isolate in virulence screens.

Three replicates of each treatment combination were used. Experimental design and procedure were otherwise identical to those used in the isolate virulence screens.

Putative differential lines. It was observed that certain double-haploid microspore-derived *B. napus* breeding lines (Table 2.1) appeared to react differentially to the virulent Canadian *L. maculans* isolates used in routine screening procedures (Stringam, G.R. and Bansal, V.K., unpublished data). This observation led to the hypothesis that further variability for virulence existed within aggressive western Canadian isolates of the fungus. Examination of these data indicated that isolates LMM89-7 and LMA89-77 gave the largest magnitude difference in interaction phenotype when inoculated onto the putative differential lines. The putative differential lines were, therefore, challenged with these two isolates. In addition, susceptible lines as well as Shiralee and Maluka-derived DH lines known to carry cotyledon resistance (Table 2.1) were included as controls. Four replicates of each treatment combination were combined in a 2 isolate × 16 genotype factorial experiment. Symptoms were evaluated according to the 0(resistant) to 4 (susceptible) scale of Stringam et al. (1992) (Table 3). A rating of 0 or 1 indicates host resistance, 2 is intermediate, and 3 or 4 indicate host susceptibility on the 0-4 scale. The method of calculating disease severity values (Equation 1) was modified accordingly by applying a maximum category value of I=4.

Standard errors (SE) of disease severities were calculated by the method used by Zhang et al. (1987) modified for use with percent disease severities (Equation 2):

$$(2)\ \mathrm{SE} = \sqrt{\frac{j - \left(\sum_{i=1}^{i} i \; fj \; / \sum_{i=1} fj \right)}{I2 \left(\sum_{i=1}^{i} fj \right) \left(\sum_{i=1}^{i} fj - 1 \right)}} \quad (100)2$$

Where i, f_j and I are the same variables used in calculating disease severities (Equation 1). All other experimental details were the same as those described above in reference to the virulence screen confirmation experiment.

Colony morphology and growth rate on V-8 juice agar. Agar plugs (ca 5 mm) carrying active mycelium were placed at the center of agar plates and incubated at approximately 170 μE m^{-2} sec^{-1} and 22° C under a 16 h photoperiod.

The following morphological characteristics were evaluated:

1. Diameter: Taken as the maximum possible distance through the center of each colony.
2. Aerial mycelium: Mycelium present on the surface of agar.
3. Pycnidium formation: Isolates classified as either producing few or numerous pycnidia.
4. Margin: The margin of each colony was classified as being either regular or irregular. The former type were smoothly circular in outline with a few sectors, while the latter type had a jagged outline, often with several sectors.
5. Mycelium color: The color of mycelium on the upper colony surface was recorded.

Colony diameters were measured 5 and 9 days after inoculation. Colony morphology parameters were also recorded at these times. The experiment was repeated once.

Growth rate on modified Czapek's agar. Differences among isolates with respect to colony expansion rates were also evaluated on 2% (w/v) Czapek's agar (Difco) amended with 1 g L^{-1} Difco yeast extract (CZY). Colonies were grown from CZY agar plugs placed onto the center of 150 × 50 ml Petri plates filled with 60 ml of CZY and covered with cellophane gel dryer membrane backing sheets (Bio-Rad Laboratories, Richmond, CA; Cat. No. 165-0959). Plates were incubated for 21 days under constant light at ambient temperatures.

Data consisted of five randomly placed radii originating at the center of each plate. One plate per isolate was used, and the entire procedure was repeated twice. Data were analyzed as a randomized complete block design (RCBD), with blocking over time. These data were collected as part of the pigment production experiment described below.

Differentiation of Isolates by Germ-tube Extension Rates. Conidial germ-tube growth rates were determined using the method of Petrie (1988). Conidiospore suspensions were prepared from actively sporulating colonies by transferring a small amount of pycnidial exudate into 10 ml of sterile distilled water. The spore density of the resulting suspensions was not fixed. Suspensions were transferred and spread onto 2% water agar (WA) with a sterile loop. The WA plates did not contain any antibiotics. Adequate separation of conidia was assured by restreaking at right angles. Inoculated plates were incubated at ca. 21° C for 40 to 48 hours in darkness. Measurements were made using an eyepiece micrometer mounted in a compound microscope (×10).

A randomized complete block design was used, with three blocks over time. Ten randomly chosen germ-tubes per plate were measured per plate by each of two observers.

Spectrophotometric analysis of *in vitro* pigment production. Pigment production of *L. maculans* isolates was determined by comparison of relative absorbances of filtered culture media. *L. maculans* cultures were grown in 10 ml aliquots of modified Czapek's broth (CZYB) consisting of Czapek's broth (Difco) amended with 1 g L^{-1} yeast extract (Difco) in 18×150 mm test tubes. The tubes were inoculated with 7 mm V8A agar plugs taken from 7-day-old cultures and incubated at ambient temperature (ca. 21 °C) for 49 days on an orbital shaker (Lab-Line Instruments Inc., Melrose Park Ill.; Model

3520) at 200 rpm. Growth of all cultures was stopped at the end of the incubation period by freezing.

Culture filtrates were produced by sequential vacuum filtration of broth cultures through filter paper (Whatman No. 1) and 0.22 µm filters (Millipore Corp., Bedford, Massachusetts; Cat No. GSWP 025 00). Relative absorbances of the filtrates were measured on a Cary 219 Ultraviolet/Visible Spectrophotometer (Varian Associates Inc., Palo Alto, California) at a previously determined wavelength of 405 nm (Figure 2.3) using filtrates from uninoculated control tubes as reference samples.

A completely randomized experimental design was used. Four tubes were prepared for each isolate. Uninoculated tubes were included as control treatments.

Examination of data revealed a non-normal distribution of absorbance and non-homogeneous error variances, thereby violating the basic assumptions of ANOVA (Steel & Torrie 1980). Analysis of variance was therefore performed on rank-transformed relative absorbance values. This approach yields results equivalent to the non-parametric Kruskal-Wallis test for *k* independent samples (Conover & Iman 1981).

Image analysis of *in vitro* pigment production. *L. maculans* colonies grown on CZY agar overlain with cellophane as described above were removed from agar by peeling-off the membranes from the surface after colony radius measurements had been taken.

Images of the upper agar surface of each plate were then captured, digitized and analyzed using a monochrome video camera fitted with a Pentax F-SMC 28mm lens set at *f*8.0 and RS12 (Eidetic Digital Imaging Ltd ,Version 3.0) image analysis software. Each digitized image was composed of 245760 pixils covering approximately 285 cm^2.

Image density was arbitrarily divided into four classes and assigned the colors red (class I), green (class II), blue (class III) and yellow (class IV). Each class corresponds to a range of pigmentation intensity, classes I and IV being the most and least intensely stained categories, respectively. The software was used to calculate the area covered by each pigment class, or density slice class area (DSCA) for each image.

A pigmentation index value (PI) was calculated for each inoculated plate by multiplying the DSCA for each pigmentation class by a weight value specific to that class. The value obtained was divided by colony area as a control against differences in growth rate among isolates. Pigmentation index may be expressed as:

$$(3)\quad PI_i = \left[\sum_{j=1}^{4}(w_j)(DSCA_j)\right]\left[R_i\right]^{-1}$$

where R is the mean colony radius of the *i*th Petri plate and DSCA and w are the density slice class area and the weight of the *j*th pigment class, respectively. The weight values were determined by:

$$(4)\quad w_j = -0.4405x_j + 112.3348$$

where x is the upper classification value of the *j*th pigment class. Equation 4 adjusted w_I and w_{IV} values to 100 and 0, respectively, as class I represents the most intensely pigmented areas, and class IV corresponds the unpigmented areas of each plate. The classification values corresponding to pigment classes I to IV as entered into the image analysis software were 1 to 28, 29 to 65, 66 to 86 and 87 to 255. The x values were therefore 28, 65, 86 and 255 for pigment classes I to IV, respectively. The same classification values were used in each repetition of the procedure.

A randomized complete block experimental design with three blocks over time was used. Pigment indices were found to differ significantly from a normal distribution and had non-homogeneous error variances. The rank-transformation method of Conover and Iman (1981) was therefore applied to image analysis data.

Cluster analysis. The relationships among *L. maculans* isolates were visualized through the use of hierarchical cluster analysis. Isolates were clustered by application of Ward's minimum variance method (Ward, 1963) to mean disease severities and all morphological parameters. Parameters assigned positive (+) or negative (-) ratings (Table 2.16) were converted to scores of 1 or 0, respectively, to permit statistical analysis. All dependant variables were then standardized to a mean of 0 and a standard deviation of 1 to remove scale and location attributes. Standardized data were submitted to the CLUSTER procedure in SAS (SAS Institute, Cary, NC) for analysis.

Results

Isolate virulence screens. *Leptosphaeria maculans* isolates were divisible into two categories on the basis of virulence on *B. napus* cv. Westar cotyledons. The first category consisted of twenty-two isolates which caused only mild symptoms on all cultivars tested (Table 2.4). The second category consisted of the remaining isolates, all of which caused severe symptoms on Westar. This category could be further subdivided into three groups. The first of these groups consisted of isolates causing only mild symptoms on cotyledons of Glacier, Quinta and Maluka (Table 2.5). The second group was comprised of nineteen Canadian isolates which caused severe reactions on cotyledons of the Australian blackleg resistant cultivar Maluka as well as those of Westar, and mild reactions on Glacier and Quinta (Table 2.6). Finally, one isolate, LMG90-112, caused severe symptoms on *B. napus* cv. Glacier cotyledons, and was therefore judged to be the sole member of a third group among the aggressive *L. maculans* isolates (Table 2.6).

Virulence screen confirmation experiment. The results obtained were similar to those from the virulence screens. Wilcoxon's signed rank test gave no significantly different disease severity values when isolates within cultivars were considered (Table 2.7). This was true when the rank of disease severity values as well as the severity values themselves were compared with the equivalent values obtained from screening data.

Analysis of variance showed highly significant genotype, isolate and interaction effects (Table 2.8). Comparisons among main factor means were performed at each level of the interacting main effects factor, since the genotype × isolate interactions were significant (Petersen 1977). Single degree-of-freedom contrasts were, therefore, carried out among isolate means within each cultivar, and multiple comparisons were made among genotypes within each isolate.

Single degree of freedom contrasts showed highly significant differences in disease severity between aggressive and non-aggressive isolates within all test cultivars (Table 2.9). When the aggressivity of the virulent Canadian isolates were compared with that of the virulent German isolate LMG90-112, highly significant effects were found with respect to the cultivars Westar, Quinta and Glacier, but not for Maluka. LMG90-112 produced less severe symptoms than Canadian isolates when inoculated onto cotyledons of Westar. In contrast, cankers on cotyledons of Quinta and Glacier were most severe when seedlings were challenged with the German isolate (Tables 2.9 and 2.10).

No significant cultivar effect was discernable among the weakly virulent isolates, since these isolates gave uniformly low disease severity values (Table 2.10). On the other hand, the Canadian virulent isolates produced significantly higher disease severity values on Westar than on Quinta and Glacier. This was also true for Maluka, except when considering the isolate Leroy, which gave a disease severity not significantly different from that obtained on Westar.

A distinct virulence profile was revealed for LMG90-112 (Table 2.10), which gave moderate disease severity values on all cultivars. This is in contrast with virulence screening data for this isolate, where susceptible interactions were observed on Westar and Quinta only (Table 2.6). This may indicate that Maluka/LMG90-112 interaction phenotypes may be sensitive to environmental effects.

Confirmation of virulence towards *B. napus* cv. Maluka cotyledons. The isolates tested were found to be virulent toward *B. napus* cv. Maluka. Analysis of variance of transformed data failed to show statistically significant isolate or cultivar × isolate interactions (Table 2.11), indicating consistent aggressivity and a lack of specific interaction among the isolates tested. Of the cultivars tested, Westar was the most susceptible across all isolates (Table 2.12). Maluka was not significantly less susceptible than Westar, with the exception of isolate LMS89-60 and "Leroy" (Table 2.12). All isolates scored less than 50% disease severity on the blackleg resistant cultivar Shiralee.

Putative differential lines. Analysis of variance indicated significant main and interaction effects (Table 2.13). Examination of control genotypes by pairwise comparisons (Table 2.14) showed significant isolate effects when resistant controls were compared with susceptible lines.

Comparisons of mean disease severities obtained from inoculation of the the ten putative differential lines with isolates LMM89-7 and LMA89-77 are given in Table 2.14. A range of disease severities was obtained but 91-479 was the only line to give a differential reaction.

Colony morphology and growth rate on V-8 juice agar. Most weakly virulent isolates of *L. maculans* were distinguishable from virulent isolates of the fungus on the basis of colony morphology on V8A (Table 2.15). Weakly virulent isolates tended to produce a floccose, darkly pigmented mycelium, and formed pycnidia infrequently (Figure 2.4). Virulent isolates, by comparison, produced a sparse, appressed, whitish mycelium and readily formed large numbers of pycnidia (Figure 2.5).

Variations among isolates with respect to colony margin, however, did not correlate well with virulence type (Table 2.15). Expression of morphological characteristics was generally consistant among subcultures of each isolate, although instability in the form of sectoring or variable expression of traits over replicates of the experiment did occur in a few cases (Figure 2.5).

One weakly virulent isolate, LMM89-1, exhibited morphological characteristics markedly different from other isolates of the same virulence type. This isolate more closely resembled virulent isolates in that it sporulated heavily and produced a scant white mycelium.

Weakly virulent isolates of *L maculans* could also be discriminated from virulent isolates on the basis of growth rate on V8A. Most weakly virulent isolates grew at a significantly greater rate than virulent isolates (Table 2.16).

Growth rate on modified Czapek's agar. The mean growth rate of weakly virulent isolates of *L. maculans* was significantly greater than that of virulent isolates at the 99% confidence level (Table 2.17). When data were inspected for significant differences among individual isolates, however, it was found that most isolates did not differ from one another with respect to growth rate. Only a few isolates represented exceptions to this observation; LMM89-1 exhibiting a particularly low rate of colony expansion.

Differentiation of Isolates by Germ-tube Extension Rates. The *L. maculans* isolates studied were found to fall into one of two groups on the basis of germ-tube extension rate. The first group consisted of isolates of the pathogen whose conidia produced germ-tubes in excess of 300 μm in 40-48 hours (Table 2.18). These isolates were all of the weakly virulent strain. The second group consisted of those isolates which produced shorter germ-tubes (<300 μm). The majority of these isolates were highly virulent toward the cotyledons of *B. napus* cv. Westar.

No well-defined subdivisions within either virulent or weakly virulent isolates were apparent. Although significant differences were found among isolates within these groups, such differences were small compared to the differences between virulent and weakly virulent strains of the pathogen.

Spectrophotometric analysis of *in vitro* pigment production. *L. maculans* isolates grown in CZY broth produced pigments ranging from pale yellow to dark brown in color, with some cultures remaining almost clear. Quantitative measurement of pigment intensity revealed that low aggressivity corresponded with staining of media among the isolates tested. F-tests performed on the ranks of relative absorbances showed that virulent isolates of *L. maculans* produced significantly less pigment *in vitro* than weakly virulent isolates at the 99% confidence level (Figure 2.6). Additional analysis performed separately on data from virulent and weakly virulent isolates revealed highly significant treatment effects, indicating the presence of a range of pigment production within each virulence type (Tables 2.19 and 2.20). Although isolates clustered into two distinct groups on the basis of pigment production in CZY broth, no relationship between culture filtrate absorbance and disease severity was evident within either of the two groups (Figure 2.7).

A large amount of dispersion existed about mean relative absorbance values among replicates of individual isolates, particularly with respect to weakly virulent isolates. The most extreme examples of this dispersion among the latter type were isolates 34 and 96, which gave absorbances ranging from 2.213 to 7.232 and 1.097 to 5.244, respectively (Table 2.19). Equivalent examples from among virulent strains were isolates 9 and 29, whose culture filtrates gave relative absorbance values ranging from 1.012 to 2.002 and 0.120 to 1.180, respectively (Table 2.20).

Image analysis of *in vitro* pigment production. Discrimination between different levels of pigment production by *L. maculans* was possible through quantification of image density. Density slice classification of digitized images (Figure 2.8) showed highly pigmented plates produced high proportions of Classes I (red), II (green).and III (blue) density slice class areas (Figure 2.8b). Such plates produced relatively high pigment indices. Moreover, lightly colored or unpigmented plates produced relatively large Class IV (yellow) areas. Pigment indices associated with these plates were comparatively high.

Staining of CZY agar by *L. maculans*, as measured by pigment index, was greatest among weakly virulent isolates. Analysis of variance of rank-transformed pigment indices showed that the pigment index of weakly virulent isolates was significantly higher than that of virulent isolates at the 99% confidence level (Figure 2.9). Individual isolates within the weakly virulent group varied widely with respect to mean pigment index (Figures 2.9 & 2.10). This was not true with respect to virulent isolates.

No statistically significant differences were found, however, when pigment indices of individual isolates within weakly virulent (Table 2.21) and virulent (Table 2.22) subclassifications were compared. Pigment production of individual isolates varied widely between repetitions of the procedure, producing large standard errors; a particularly frequent occurrence among weakly virulent isolates.

Cluster analysis. *Leptosphaeria maculans* isolates formed two unequally sized groups at a low level of similarity (Figure 2.11). The larger cluster consisted for the most part of isolates that produced severe interaction phenotypes on *B. napus* cv. Westar cotyledons. Conversely, the smaller group consisted mainly of non-aggressive isolates. Sub-clusters which formed within each of the two main groups appear to have been based on other virulence characteristics and modified by morphological parameters. In particular, membership in one cluster in the larger group consisted entirely of isolates causing severe symptoms on cotyledons of *B. napus* cv. Maluka (Isolates LMA89-81, LMM89-37 LMS89-25, LMS89-60, LMS89-33, VBL89-97 LMS89-17, LMS89-75, LMS89-14, LMS89-30, LMS89-19, VBL89-95 and LMA89-65). The weakly virulent, slow growing and heavily sporulating isolate LMM89-1 was associated with the aggressive isolates, but formed its own cluster within this group.

Examination of results revealed no grouping of isolates which could be associated with geographic distribution of isolate collection sites. The only exception to this observation was an isolate collected near Paderborn, Germany, LMG90-112, which formed a single-member cluster within the the larger body of aggressive isolates. Western Canadian isolates collected in proximal locations however, failed to cluster together.

Discussion

The results of this study confirm those of previous workers since western Canadian isolates were found to be divisible into two groupson the basis of reaction on *B. napus* cv. Westar cotyledons (Koch et al. 1991, McGee & Petrie 1978, Mengistu et al. 1991). Non-aggressive isolates (those causing no or only slight symptoms on Westar cotyledons) appear to correspond to weakly virulent strains as described by McGee and Petrie (1978) and to the "Puget Sound" strain of Pound (1947). The differences between the two types were quite distinct as no isolates of intermediate virulence were discovered.

The lack of intermediate types supports speculation that the two *L. maculans* strains are distinct species, or at least do not constitute sexually intermating populations. All attempts at sexual matings have been unsuccessful (Delwiche 1980, Petrie & Lewis 1985, Venn 1979), although crosses between isolates within the same pathogenicity type have succeeded. Johnson and Lewis (1990) were able to differentiate virulent from weakly virulent strains on the basis of DNA polymorphism. Taylor et al. (1991) obtained similar results when electrophoretic karyotypes of the two types were compared.

All other isolates were members of the aggressive strain which has been spreading through canola acreage since 1978 (Petrie 1978). All these isolates behaved in a manner consistent with that of PG2 (Koch et al. 1991) since both of the differential isolates Quinta and Glacier were less severely affected than Westar. These findings are consistent with those of Kutcher (1990) and Mengistu *et al.* (1991) who found that all aggressive *L. maculans* isolates originally collected in western Canada behaved in a similar manner on this particular set of differential cultivars.

A substantial number of the aggressive isolates caused severe symptoms on the cotyledons of the cultivar Maluka. This is surprising since this cultivar is resistant in field trials, and since doubled-haploid lines derived from crosses of Maluka with susceptible genotypes yield resistant and susceptible progeny in a 1:1 ratio (Stringam *et al.* 1992) indicating single gene inheritance of resistance. This may indicate that Maluka is not homozygous for reisitance. Alternately, variation for virulence among virulent western Canadian isolates of *P. lingam* not detectable by the differential cultivars Westar, Quinta and Glacier may exist. A possible mechanism for this variability could be a gene-for-gene system in which the cultivar Maluka carries genes conditioning susceptibility that are not present in Quinta or Glacier, and that these genes are matched by corresponding pathogen genes conditioning virulence on Maluka. Some evidence in support of this model exists. Rimmer & van den Berg (1992) state that two genotypes are found within virulent western Canadian isolates, although both fungal genotypes produced the same interaction phenotype on Westar. It is possible that these two genotypes produce distinct interaction phenotypes when infecting Maluka. No evidence can be found however, to support the notion that the Canadian isolates collected for this study are divisible into two distinct pathogenicity groups or races. Although the isolates were placed into two separate groups on the basis of reaction on Maluka cotyledons, the range of responses across aggressive isolates with respect to this parameter was in fact continuous. A continuous distribution of disease response would explain non-significant differences between some Maluka-aggressive and Maluka-nonaggressive isolates.

The differential response obtained for isolates LMM89-7 and LMA89-77 with respect to the microspore-derived *B. napus* line 91-479 appears to argue against general differences in aggressivity among virulent isolates. However, closer examination of the pedigree of 91-479 revealed that this line was derived from a spontaneous diploid. It may be possible that this line is the progeny of an unreduced gamete. The line 91-479 can be considered neither homozygous nor homogeneous for resistance. Consequently, no specific interactions can be attributed to isolates LMM89-7 and LMA89-77 with respect to this line.

It may, therefore, be inferred that no further variation in specific virulence conditioning genes exists among the virulent *L. maculans* isolates collected in western Canada, although the isolates vary in general aggressivity. Thus, it appears that *L. maculans* carries a few major cultivar-specific virulence genes supplemented by an unknown number of non-specific minor genes. These minor genes appear to have only small individual and cumulative effects. Moreover, the dearth of isolates expressing intermediate aggressivity towards *B. napus* cv. Westar cotyledons suggests that this trait is under monogenic control. This is in contrast with fungi such as *Verticillium dahliae*, in which pathogenicity is under polygenic control. A consequence of this is that wild-type isolates of *V. dahliae* exhibit a normally-distributed continuum of virulence on susceptible tomato cultivars (O'Garro & Clarkson 1992).

Selection for resistance to major *L. maculans* virulence genes would produce resistance of the greatest magnitude because of the presence of major resistance genes. Selection for resistance toward western Canadian isolates should be relatively simple since this trait appears to be controlled by a monogenic dominant gene (Stringam et al. 1992). This does not, however, protect against the possibility of catastrophic loss via pathogen mutation or importation of new virulence genes. Isolate LMG90-112, collected near Paderborn, Germany, was virulent on all *B. napus* cultivars tested, although Glacier appeared to be slightly less susceptible. No Canadian isolates share this virulence profile. These findings are consistent with those of Mengistu et al. (1991), who found that isolates virulent on Quinta and Glacier were rare or absent in Saskatchewan or Manitoba, but common in Europe. The virulence profile of LMG90-112 may be of concern to breeders interested in incorporating blackleg resistance into *B. napus* lines, particularly if virulence on Glacier and Quinta cotyledons in greenhouse tests can be extrapolated to field results. It would therefore be prudent to deploy resistance genes effective against virulent strains not currently common in western Canada.

The results of this study also confirm those of previous workers who found that *L. maculans* isolates have one of two colony morphologies; a floccose, rapidly growing form with a pigmented mycelium which produces few pycnidia, and a freely sporulating, slowly growing form with an appressed mycelium (Delwiche 1980, Hill et al. 1984, Petrie 1988, Pound 1947). As also observed in these previous studies, the former type of colony morphology was found to be associated with the weakly virulent strain, whereas the latter type was associated with virulent isolates.

Colony morphology cannot be considered to be a reliable predictor of virulence, however, since not all isolates conformed to the expected morphologies. A striking example of this was isolate LMM89-1, which consistently caused only mild symptoms on cotyledons of *B. napus* cv. Westar, despite physical characteristics more consistent with the aggressive strain of *L. maculans*. Similar, though less striking examples of cultures which behaved in an unexpected manner were found among virulent *L. maculans* isolates as well. Isolate LMM89-6, for example, produced abundant mycelium and few pycnidia in culture on V8 juice agar.

Considerable variability was observed among isolates of *L. maculans* with respect to growth rate on both V8 juice and modified Czapek's agars, although the mean growth rate of isolates of the non-aggressive strain was significantly greater than that of the aggressive strain on both media. It is, therefore, apparent that while colonies of aggressive isolates in general expand at a lower rate than non-aggressive isolates, the general case cannot be applied to individual isolates. A similar observation was made by Pound and Calvert (1948) who, working with *L. maculans* isolated from cabbage seed, found isolates of the pathogen which resembled virulent "eastern" strains of the pathogen in culture, but the weakly virulent 'Puget Sound" strain in pathogenicity.

Among the morphological traits measured, germ-tube extension rate appeared to differentiate between aggressive and non-aggressive strains of *L. maculans* most efficiently. Very few of the weakly virulent isolates produced germ-tubes of less than 300 µm in length, and none of the virulent isolates produced germ-tubes in excess of that figure. Thus, the results of this study are similar to those of Petrie (1988) who found a similar, well defined correspondence between germ-tube extension rate and virulence. The results of this study differ from Petrie's (1988) results, however, in that both virulent and weakly virulent isolates produced shorter germ-tubes in the latter study. This may be due to the inclusion by Petrie (1988) of antibiotics in the agar plates used in that study. Alternatively, the growth rate differences between the two studies may be due to differences in environmental conditions, or to differences in spore concentrations. The need for the inclusion of a standard control isolate is underscored if either or both of the latter two cases are correct, since the discriminative ability of the test stems from relative differences in growth rates of aggressive and non-aggressive strains.

Nevertheless, the germ-tube test is an effective predictor of isolate aggressivity. A good example of this is the response of LMM89-1. This weakly virulent isolate responded in a manner indicative of the aggressive strain of *L. maculans* with respect to all morphological parameters, excepting conidiospore germ-tube growth rate. This result is of particular interest since colonies of LMM89-1 expanded at a particularly slow rate on both V8 juice and modified Czapek's agar media. This shows that germ-tube extension rate and *in vitro* growth rate are distinct traits in *L. maculans*; that is, measurement of conidial germ-tubes is not simply a rapid method of determining an isolate's growth rate.

Production of pigment in culture has long been cited as a character capable of differentiating between aggressive and non-aggressive isolates of *L. maculans*. Pound (1947) stated that weakly virulent "Puget Sound" isolates of the fungus were distinguishable partly on the basis of production of water soluble yellow to brown pigment in several media. This correlation between weak virulence and pigment production has since been confirmed by other workers (Delwiche 1980, Koch et al. 1989, McGee & Petrie 1978). However, no quantitative measurements of *in vitro* pigment production have been described prior to the present study.

The methods used in the present study were designed to allow discrimination between and among virulent and weakly virulent isolates through quantitative analysis of pigment intensity. Previous workers (Koch et al., 1989, McGee & Petrie 1978) found that pigmentation varies from light yellow to dark brown, with weakly virulent isolates producing pigment of the greatest intensity. Quantitative analysis of pigment production confirms the positive relationship between weak virulence and copious pigment production. The present study also shows, however, that intensity of pigmentation is a highly variable trait, even among subcultures of the same isolate. Pigment production, therefore, is not a good predictor of virulence since some aggressive isolates produced appreciable amounts of pigment, and since there was a large amount of overlap between virulent and weakly virulent isolate pigment production means, regardless of the quantification method used.

Image analysis data showed unnessecarily large error variances, which were probably due partly to variations in culture growth conditions. To correct this, all replicates of future experiments should be run at once to compensate for temporal variations in environmental conditions. In addition, within-isolate variability in image density caused by block-to-block variations in light conditions also contributed to experimental error. Future improvements to the image analysis protocol should, therefore, include changing class limit variables for each run of the procedure.

Quantitative measurement of *in vitro* pigment production by visible-range spectrophotometry, however, gave similar results to those obtained through image analysis. The similarity of results obtained through the two approaches suggests that any particular isolate of *L. maculans* is capable of modulating its production of pigment over a wide range of intensities in response to relatively small variations in its abiotic environment. This would provide an explanation for the wide range of responses seen both among replicate cultures of the same isolate, and non-significant or continuously distributed differences between isolates of the same *L. maculans* strain (virulent or weakly virulent).

This broad range of pigment production prevented further discrimination among isolates within either strain. The correlation between virulence and pigmentation, however, holds true as a general statement.

Table 2.1. *Brassica napus* lines and cultivars used in cotyledon tests.

Line or cultivar	Description
Differential cultivars:	
Westar	Susceptible, spring-type
Quinta	Resistant, winter-type
Glacier	Resistant, winter-type
Other cultivars:	
Maluka	Partially-resistant, spring-type
Profit	" "
Shiralee	" "
†Putative differential lines:	
91-479	DH from F_1 Shiralee/Susceptible
90-4977	DH from F_1 Maluka/Susceptible
91-15008	" " " " "
91-15019	" " " " "
91-15031	" " " " "
91-15041	" " " " "
91-15044	" " " " "
91-15096	" " " " "
91-15097	" " " " "
91-15100	" " " " "
†Resistant accessions:	
91-4856	DH from F_1 Shiralee/Susceptible
91-15066	" " " " "
91-15094	DH from F_1 Maluka/Susceptible
†Susceptible accessions:	
90-3045	DH from F_1 Resistant/Susceptible
90-4301	" " " " "

†Microspore-derived doubled-haploid.

Table 2.2. *Leptosphaeria maculans* isolates used in this study.

Isolate	Collection site	Isolate	Collection site
LMM89-1	Riding Mountain, Man.	LMM89-48	Oakner, Man.
LMM89-2	Westbourne, Man.	LMM89-49	Star City, Man.
LMM89-3	Neepawa, Man.	LMS89-51	Cudworth, Sask.
LMM89-4	Headingly, Man.	LMS89-52	Cudworth, Sask.
LMM89-5	Elgin, Man.	LMS89-53	Meacham, Sask.
LMM89-6	Pilot Mound, Man.	LMS89-54	Kelvington, Sask.
LMM89-7	Darlingford, Man.	LMS89-55	Hazel Dell, Sask.
LMM89-8	Deloraine, Man.	LMS89-58	Glaslyn, Sask.
LMM89-9	Wroxton, Man	LMS89-60	Marshall, Sask.
LMM89-11	Garrick, Man.	LMS89-61	Cutknife, Sask.
LMS89-12	Choiceland, Sask.	LMA89-64	Innisfree, Alta.
LMS89-14	Prince Albert, Sask.	LMA89-65	Mannville, Alta.
LMS89-17	Alvena, Sask.	LMS89-75	Armley, Sask.
LMS89-18	Wakaw, Sask.	LMA89-77	Andrew, Alta.
LMS89-19	Lake Lenore, Sask.	LMA89-78	Andrew, Alta.
LMS89-22	Leroy, Sask.	LMA89-79	Mundare, Alta.
LMS89-24	Norquay, Sask.	LMA89-81	Sedgewick, Alta.
LMS89-25	Wynyard, Sask.	LMM89-82	Hamiota, Man.
LMS89-26	Mikado, Sask.	VBL89-95	A.E.C.[†]
LMS89-27	Stockholm, Sask.	VBL89-14	A.E.C.
LMS89-29	Lashburn, Sask.	VBL89-72	A.E.C.
LMS89-30	Cutknife, Sask.	VBL89-20	A.E.C.
LMS89-31	North Battleford, Sask.	VBL89-97	A.E.C.
LMS89-32	Marsden, Sask.,	BL87-9	A.E.C.
LMS89-33	North Battleford, Sask.	BL87-10	A.E.C.
LMS89-34	North Battleford, Sask.	LMA89-91	A.E.C.
LMS89-35	Rockhaven, Sask.	AVBL89-23	A.E.C.
LMS89-36	Aberdeen, Sask.	AVBL89-96	A.E.C.
LMM89-37	Dauphin, Man.	BL88-22(4)	A.E.C.
LMA89-40	Legal, Alta.	Leroy	Leroy, Sask.[‡]
LMA89-41	Sherwood Park, Alta.	BL-4wv	A.E.C.[†]
LMA89-42	Vegreville, Alta.	LMG90-112	Paderborn, Germany[§]
LMA89-43	Vegreville, Alta.	LMA91-117	Lloydminster, Alta.[††]
LMA89-44	Vegreville, Alta.	LMA90-3356	Ellerslie, Alta.[§]
LMA89-45	Vermillion, Alta.		

[†] Provided by Dr. Prem Kharbanda, Alberta Environmental Centre, Vegreville, Alberta.

[‡] Provided by Dr. G.A. Petrie, Agriculture Canada, Saskatoon, Saskatchewan.

[§] Provided by Dr. J.P. Tewari, Department of Plant Science, University of Alberta.

[††] Provided by Dr. Vipan K. Bansal, Department of Plant Science, University of Alberta.

Table 2.3. Interaction Phenotypes (IP) of *Brassica napus* cotyledons inoculated with *Leptosphaeria maculans*.

IP class	Interaction Phenotype
0-9 scale†:	
0	No infection.
1	Slight necrosis around wound only.
3	Non-spreading lesion with blackening and/or tissue collapse
5	Sharply defined lesion with black margin.
7	Spreading lesion without a black border, often with indistinct margin.
9	Pycnidia within lesion.
0-4 scale‡ :	
0	No infection.
1	Slight necrosis around wound only.
2	Small lesion.
3	Spreading, non-sporulating lesion.
4	Pycnidia within lesion.

†Adapted from Williams (1985).
‡Stringam et al. (1992).

Table 2.4. Virulence profiles *Leptosphaeria maculans* isolates causing disease severities of less than fifty percent on *Brassica napus* cv. Westar cotyledons.

	Disease reaction on cultivar†:			
Isolate	Westar	Quinta	Glacier	Maluka
LMM89-1	12.5	11.1	11.1	11.1
LMM89-2	11.1	13.9	24.8	11.8
LMM89-5	15.0	15.0	15.0	15.0
LMM89-11	27.1	19.9	14.8	11.8
LMS89-12	16.0	14.6	14.0	20.8
LMS89-18	17.4	23.1	18.8	13.2
LMS89-24	24.3	25.7	20.1	11.1
LMS89-26	20.1	21.3	16.2	12.5
LMS89-34	11.8	11.1	11.1	11.1
LMS89-36	25.7	16.0	20.1	74.3
LMA89-40	22.5	23.5	11.8	11.1
LMA89-41	33.6	13.2	13.2	18.8
LMA89-45	25.2	13.9	24.3	20.1
LMS89-54	15.1	14.6	18.1	14.6
LMS89-58	11.8	11.8	11.1	11.8
LMA89-78	18.9	12.5	11.1	18.8
LMA89-79	11.8	11.1	11.1	22.2
LMA89-91	38.4	15.6	21.8	22.2
AVBL89-23	32.7	13.9	13.9	52.6
AVBL89-96	18.1	12.5	11.8	40.0
BL-4wv	17.0	11.1	11.8	11.1
BL88-22(4)	11.8	13.0	11.1	11.1

†Percent disease severity, based on interaction phenotype.

Table 2.5. Virulence profiles of virulent *Leptosphaeria maculans* isolates causing disease severities greater than fifty percent on *Brassica napus* cv. Westar cotyledons.

	Disease reaction on cultivar†:			
Isolate	Westar	Quinta	Glacier	Maluka
LMM89-4	95.6	31.0	50.7	33.9
LMM89-6	100.0	13.9	22.9	11.1
LMS89-22	99.3	11.8	21.8	22.2
LMS89-27	100.0	11.5	21.3	34.0
LMS89-29	98.6	13.0	22.9	11.1
LMS89-31	86.4	14.3	30.5	11.8
LMS89-32	94.4	16.3	27.8	21.5
LMS89-35	96.5	37.5	30.9	14.6
LMA89-42	100.0	18.9	18.1	19.6
LMA89-43	95.2	11.8	32.5	11.1
LMA89-44	92.4	12.5	19.4	11.1
LMM89-48	97.9	11.9	19.7	11.1
LMM89-49	97.6	11.1	24.3	37.5
LMS89-51	100.0	11.1	22.2	12.5
LMS89-52	99.3	13.2	13.9	28.5
LMS89-53	100.0	12.5	22.2	11.8
LMA89-64	66.1	18.8	11.1	14.6
LMA89-77	88.9	42.8	21.4	11.8
LMA89-81	100.0	23.4	20.1	12.5
LMM89-82	96.3	32.6	16.7	12.5
LMA90-3356	97.2	26.0	22.2	29.0
BL87-9	100.0	13.9	15.3	18.1
BL87-10	89.6	27.8	16.7	13.9
Leroy	97.7	18.0	21.0	45.0
VBL89-14	92.4	16.7	19.4	11.1
VBL89-20	92.0	35.9	16.7	11.1

†Percent disease severity, based on interaction phenotype.

Table 2.6. Virulence profiles of *Leptosphaeria maculans* isolates causing disease severities greater than fifty percent on cotyledons of *Brassica napus* cvs. Westar and Maluka.

	Disease reaction on cultivar[†]:			
Isolate	Westar	Quinta	Glacier	Maluka
LMM89-3	100.0	11.8	13.2	80.0
LMM89-7	100.0	16.2	31.1	52.4
LMM89-8	100.0	13.9	12.5	88.9
LMM89-9	100.0	43.1	39.6	84.5
LMS89-14	99.3	15.3	13.2	67.4
LMS89-17	100.0	11.1	11.1	75.0
LMS89-19	100.0	20.1	16.4	77.1
LMS89-25	96.5	12.5	16.0	72.5
LMS89-30	100.0	19.4	14.6	61.3
LMS89-33	100.0	11.0	15.5	79.6
LMM89-37	100.0	13.2	14.6	82.6
LMS89-55	96.5	22.3	37.4	81.3
LMS89-60	100.0	11.8	13.2	77.8
LMS89-61	99.3	26.7	24.7	64.6
LMA89-65	98.5	25.4	15.3	72.2
LMA89-75	100.0	9.4	12.5	77.8
VBL89-72	96.4	43.1	20.8	81.0
VBL89-95	100.0	16.0	19.3	69.3
VBL89-97	100.0	11.1	15.3	75.3
LMA90-112	54.4	22.9	70.0	34.2

[†] Percent disease severity, based on interaction phenotype.

Table 2.7. Change in disease severity (DS) and ranking of isolate severity between data obtained from isolate virulence screens and confirmation experiments.

	Cultivar							
	Westar		Quinta		Glacier		Maluka	
Isolate	ΔDS†	ΔR‡	ΔDS	ΔR	ΔDS	ΔR	ΔDS	ΔR
LMM89-1	1.4	0.0	0.0	0.0	0.0	0.0	0.0	-0.5
LMM89-3	0.0	0.0	-13.6	0.0	-9.0	-3.0	38.8	1.0
LMS89-26	9.0	0.0	10.2	5.0	5.1	3.0	1.4	0.0
LMA89-64	-27.7	-1.0	-18.4	0.0	-7.9	-4.0	-24.1	-2.0
BL88-22(4)	0.7	0.0	1.9	2.0	0.0	0.0	0.0	-0.5
Leroy	6.0	3.0	-7.7	1.0	4.0	1.0	-5.0	-1.0
LMG90-112	-7.0	-1.0	-51.4	1.0	23.0	0	-22.9	-3.0
LMA90-3356	3.1	-1.0	-0.2	4.0	8.2	3.0	-3.6	0.0
T*	15.0 ns	13.0 ns	9.0 ns	2.0 ns	12.0 ns	16.0 ns	14.0 ns	12.0 ns

† Change in DS. ‡ Change in ranking of isolate for DS. * Wilcoxon's Signed Rank Test statistic.
ns Not statistically significant at 5% level.

Table 2.8. Analysis of variance of disease reactions of *Brassica napus* cvs Westar, Glacier Quinta and Maluka to eight† *Leptosphaeria maculans* isolates.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square	Computed F
Genotype	3	14964.0	4988.0	83.15^{**}
Isolate	7	21416.1	3059.4	51.0^{**}
Genotype × isolate	21	14028.7	668.0	11.1^{**}
Error	96	5759.1	59.9	
Corrected Total	127	56168.0		

† LMM89-1, LMS89-26, BL88-22(4), LMM89-3, LMA89-64, LMA90-3356, LMG90-112 and Leroy.

** Significant at 1% level

Table 2.9. Comparison of disease severity of eight *Leptosphaeria maculans* isolates challenging four *Brassica napus* cultivars.

	Probability > F within cultivar:			
Contrast	Westar	Quinta	Glacier	Maluka
1. Weakly virulent *vs.* Virulent isolates.	0.0001	0.0001	0.0052	0.0001
Comparisons among weakly virulent isolates:				
2. LMM89-1 *vs.* LMS89-29 & BL-4wv.	n s	n s	n s	n s
3. LMS89-26 *vs.* BL-4wv.	n s	n s	n s	n s
Comparisons among virulent isolates:				
4. LMA89-64 & LMA90-3356 *vs.* LMG90-112	0.0022	0.0001	0.0002	n s
5. LMA89-64 & LMA90-3356 *vs.* LMM89-3 & Leroy	n s	n s	n s	n s
6. LMM89-3 *vs.* Leroy	0.0006	n s	n s	n s
7. LMA89-64 *vs.* LMA90-3356	n s	n s	n s	n s

ns Not statistically significant at the 1% level.

Table 2.10. Interactions between eight *Leptosphaeria maculans* isolates and cotyledons of four *Brassica napus* cultivars.

	Disease severity †							
	Weakly virulent isolates			Virulent isolates				
Cultivar	LMM89 -1	LMS89 -26	BL86 -22(4)	LMA89 -64	LMA90 -3356	LMM89 -3	Leroy	LMG90 -112
Westar	11.1a	11.1a	11.1a	93.8a	94.1a	100a	76.3a	76.4a
Quinta	11.1a	11.1a	11.1a	37.2b	26.2b	25.4b	25.7b	74.3a
Glacier	11.8a	13.2a	11.1a	19.0b	14.0b	22.2b	17.0b	47.0a
Maluka	11.1a	11.1a	11.1a	38.7b	32.6b	41.2b	50.0ab	57.1a

† Mean of four replicates. Means within a column with the same letter are not statistically different by Tukey's studentized (HSD) range test at the 1% level.

Table 2.11. Analysis of variance of disease reactions of *Brassica napus* cvs Westar, Maluka and Shiralee to eight virulent *Leptosphaeria maculans* isolates†.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square	Computed F
Cultivar	2	32014.2	16007.1	228.9**
Isolate	7	353.3	50.4	0.72ns
Cultivar × isolate	14	1639.7	117.1	1.68ns
Error	47	3286.4		
Corrected total	70	37450.4		

† LMM89-3, LMM89-8, LMS89-33, LMS89-55, LMS89-60, LMS89-75, VBL89-72 and Leroy.

** Significant at the 1% level.

ns Not statistically significant at the 5% level.

Table 2.12. Disease severity of plants inoculated with eight *Leptosphaeria maculans* isolates virulent on *Brassica napus* cv Maluka.

Cultivar	Isolate†								
	LMM89-3	LMM89-8	LMS89-33	LMS89-55	LMS89-60	LMS89-75	VBL89-72	Leroy	Mean
Westar	100.0a	93.6a	95.3a	98.6a	100.0a	100.0a	100.0a	100.0a	98.7
Maluka	81.3a	85.3a	75.0ab	85.6ab	54.3b	85.3a	80.3ab	77.6b	77.9
Shiralee	22.6b	35.3b	29.6b	22.6b	44.6b	32.3b	47.0b	25.6c	32.5
Mean	68.0	72.3	66.6	68.0	66.3	72.5	75.5	67.7	

†Mean of three replications. Means in a column with the same letter are not statistically different by Tukey's Studentized (HSD) Range test at the 1% level.

Table 2.13. Analysis of variance of disease reactions of sixteen *Brassica napus* genotypes† to two virulent *Leptosphaeria maculans* isolates LMM89-7 and LMA89-77.

Source of variation	Degree of Freedom	Sum of Squares	Mean Square	Computed F
Genotype	15	558.6	558.6	21.98**
Isolate	1	62261.6	4150.7	168.3**
Genotype × isolate	15	1340.1	89.3	3.51**
Error	96	2440.0		
Corrected total	127			

† Ten putative differential lines, lines 91-4856, 91-15066, 91-15094, 90-3045 and 90-4301, and Profit.

** Significant at 1% level

Table 2.14. Disease severities† (DS) and their standard errors (S.E.) derived from inoculation of susceptible, resistant and putative differential DH lines with two virulent *Leptosphaeria maculans* isolates.

	LMM89-7			LMA89-77	
Line	DS	S.E.		DS	S.E.
Susceptible genotypes:					
90-3045	100.0a	(0)		100.0a	(0)
90-4301	100.0a	(0)		100.0a	(0)
Profit	98.4a	(9.7)		100.0a	(0)
Resistant genotypes:					
91-15066	25.8e	(2.4)		25.0e	(0)
91-4856	25.0e	(0)		27.3e	(9.0)
91-15094	25.8e	(4.2)		31.3e	(2.1)
Putative differential lines:					
90-4977	80.5b	(2.2)		83.6bc	(3.5)
91-479	53.2c	(5.0)	**	88.3ab	(2.2)
91-15008	52.5cd	(4.2)		62.9cd	(4.3)
91-15019	29.7e	(2.1)		36.7de	(3.2)
91-15031	26.6e	(0.1)		29.8e	(1.1)
91-15041	32.8e	(3.3)		53.1de	(4.9)
91-15044	27.3e	(1.7)		28.1e	(1.5)
91-15096	25.0e	(0)		32.8e	(2.6)
91-15097	25.8e	(0.8)		26.6e	(1.6)
91-15100	25.8e	(0.8)		25.0e	(0)

†Mean of four replicates. Means in a column with the same letter are not significantly different by Tukey's Studentized (HSD) Range.

Means separated by ** are significantly different by Tukey's Studentized (HSD) Range at the 1% level.

Table 2.15. Colony morphologies of *Leptosphaeria maculans* isolates on V-8 juice agar.

Weakly virulent isolates					Virulent isolates								
Isolate	Myc†	Pyc‡	Col§	Mar††	Isolate	Myc	Pyc	Col	Mar	Isolate	Myc	Pyc	Col	Mar
LMS89-36	+	+	-	+/-	LMS89-61	+/-	+	+/-	+/-	LMS89-33	-	+	-	+
LMS89-34	+	-	+/-	-	LMM89-3	+/-	+/-	-	+/-	LMS89-35	-	+	-	+
LMM89-2	+	-	+	+	LMM89-4	+	+/-	-	+/-	LMM89-37	-	+	-	+
LMM89-11	+	-	+	+	LMS89-32	+	+/-	-	-	LMA89-42	-	+	-	+
LMS89-12	+	-	+	-	LMM89-6	+	-	+	+	LMA89-43	-	+	-	+
LMS89-18	+	-	+	+	LMM89-48	-	+/-	-	+/-	LMA89-44	-	+	-	+
LMS89-24	+	-	+	+	LMM89-49	-	+	-	+/-	LMS89-51	-	+	-	+
LMS89-26	+	-	+	+	LMS89-58	-	+	-	+	LMS89-52	-	+	-	+
LMA89-40	+	-	+	+	LMM89-7	-	+	-	+	LMS89-55	-	+	-	+
LMA89-41	+	-	+	+	LMM89-8	-	+	-	+	LMS89-60	-	+	-	+
LMA89-45	+	-	+	+	LMM89-9	-	+	-	+	LMA89-64	-	+	-	+
LMS89-54	+	-	+	+	LMS89-14	-	+	-	+	LMA89-65	-	+	-	+
LMA89-78	+	-	+	+	LMS89-17	-	+	-	+	LMS89-75	-	+	-	+
LMA89-91	+	-	+	+	LMS89-19	-	+	-	+	LMA89-77	-	+	-	+
AVBL89-23	+	-	+	+	LMS89-22	-	+	-	+	LMA89-81	-	+	-	+
AVBL89-96	+	-	+	+	LMS89-25	-	+	-	+	LMM89-82	-	+	-	+
BL86-22(4)	+	-	+	+	LMS89-27	-	+	-	+	BL87-10	-	+	-	+
LMM89-1	-	+	-	+	LMS89-29	-	+	-	+	BL87-9	-	+	-	+
LMM89-5	-	+	-	+	LMS89-30	-	+	-	+	Leroy	-	+	-	+
					LMS89-31	-	+	-	+	VBL89-14	-	+	-	+
					VBL89-95	-	+	-	+	VBL89-20	-	+	-	+
					VBL89-97	-	+	-	+	VBL89-72	-	+	-	+

†Mycelium type: flocculent(+) or appressed (-). ‡Pycnidium density: abundant (+) or sparse (-)
§Color of Mycelium: pigmented (+) or white (-). ††Colony margin: regular (+) or irregular (-).

Table 2.16. Growth rates[†] of *Leptosphaeria maculans* isolates on V-8 juice agar.

Weakly virulent isolates			Virulent isolates					
Isolate	Mean[‡]	SE	Isolate	Mean	SE	Isolate	Mean	SE
BL86-22(4)	8.8a	0.8	LMM89-6	8.4a-d	0.2	LMS89-75	5.7f-n	0.1
AVBL89-96	8.7a-b	0.1	LMS89-26	8.2a-e	0.0	LMS89-17	5.6h-n	0.1
LMM89-11	8.6a-c	0.2	LMM89-48	6.6c-l	0.9	LMS89-30	5.6h-n	0.4
LMA89-40	8.4a-d	0.2	LMM89-49	6.6c-l	0.5	BL87-10	5.6h-n	0.1
LMA89-91	8.2a-e	0.0	LMM89-5	6.5c-l	0.5	LMM89-8	5.5h-n	0.5
LMM89-2	7.8a-f	0.3	LMS89-14	6.4d-l	0.4	LMS89-32	5.5h-n	0.5
LMA89-45	7.7a-g	0.1	LMS89-19	6.4d-l	0.2	LMS89-25	5.5i-n	0.1
LMA89-79	7.6a-h	0.4	LMM89-7	6.1e-m	0.1	LMA89-42	5.5i-n	0.3
LMA89-78	7.5a-i	0.5	LMM89-9	6.1f-m	0.5	LMA89-44	5.5i-n	0.1
LMS89-54	7.5a-j	0.3	LMA89-65	6.1f-m	0.1	LMS89-35	5.4j-n	0.1
LMA89-41	7.4a-k	0.2	LMA89-77	6.1f-n	0.0	LMS89-60	5.4j-n	0.4
LMS89-18	7.2a-k	0.2	BL87-9	6.1f-n	0.1	LMS89-29	5.3k-n	0.3
LMS89-34	7.1a-k	1.1	Leroy	6.1f-n	0.1	VBL89-14	5.2k-n	0.0
LMS89-24	6.4d-l	0.2	LMS89-27	6.0f-n	0.0	LMA89-81	5.1k-n	0.4
AVBL89-23	6.3d-m	0.1	LMS89-33	6.0f-n	0.2	LMM89-3	5.0l-n	0.0
LMS89-12	6.3e-m	0.8	VBL89-20	6.0f-n	0.5	LMS89-52	5.0l-n	0.4
LMS89-36	6.3e-m	0.3	LMS89-61	5.9f-n	0.1	LMS89-22	4.9l-n	0.3
LMS89-58	5.7f-n	0.1	LMM89-37	5.8f-n	0.4	LMS89-53	4.9l-n	0.3
LMM89-1	2.4o-q	0.2	LMS89-55	5.8f-n	0.0	LMA89-64	4.9l-n	0.1
			VBL89-95	5.8f-n	0.0	VBL89-72	4.8l-n	0.2
			VBL89-97	5.8f-n	0.1	LMM89-82	4.2m-o	0.6
			LMA89-43	5.7f-n	0.1	LMS89-31	4.0n-p	0.2
			LMS89-51	5.7f-n	0.0	LMM89-4	1.9p-q	0.5

[†] Mean increase in colony diameter (mm day^{-1}) of three replicates

[‡] Means with the same letter are not significantly different by Tukey's multiple range (HSD) test at the 1% level.

Table 2.17. Growth rates[†] of *Leptosphaeria maculans* isolates on modified Czapek's agar.

Weakly virulent isolates			Virulent isolates					
Isolate	Mean[‡]	SE	Isolate	Mean	SE	Isolate	Mean	SE
LMA89-79	4.3a	1.1	LMS89-26	2.9b-d	0.2	LMA89-65	2.2b-f	0.0
LMS89-36	3.2b	.	LMS89-61	2.6b-e	0.2	VBL89-95	2.2b-f	0.1
LMS89-54	3.2b	0.0	LMM89-8	2.4b-f	0.3	LMS89-22	2.1b-f	0.0
LMA89-78	3.2b	0.0	LMM89-49	2.4b-f	0.2	LMA89-44	2.1b-f	0.0
AVBL89-96	3.2b	0.0	LMA89-81	2.4b-f	0.1	LMA89-64	2.1b-f	0.1
BL-4wv	3.2bc	0.0	VBL89-72	2.4b-f	0.3	LMS89-75	2.1b-f	0.1
LMS89-58	3.1b-d	0.1	LMM89-4	2.3b-f	0.1	LMG90-112	2.1b-f	0.0
LMA89-91	3.1b-d	0.2	LMM89-7	2.3b-f	0.2	BL87-10	2.1b-f	0.1
AVBL89-23	3.1b-d	0.2	LMM89-37	2.3b-f	0.0	VBL89-14	2.1b-f	0.0
LMA89-11	3.0b-d	0.1	LMA89-42	2.3b-f	0.0	VBL89-20	2.1b-f	0.1
LMS89-24	3.0b-d	0.2	LMM89-48	2.3b-f	0.2	VBL89-97	2.1b-f	0.1
LMS89-34	2.9b-d	0.3	LMA89-77	2.3b-f	0.1	LMM89-3	2.0b-f	0.1
LMA89-40	2.9b-d	0.2	LMM89-82	2.3b-f	0.1	LMM89-6	2.0b-f	0.1
LMA89-41	2.9b-d	0.2	LMA91-117	2.3b-f	0.1	LMS89-14	2.0b-f	0.1
LMS89-12	2.8b-d	0.2	LMA90-3356	2.3b-f	0.2	LMS89-27	2.0b-f	0.1
LMS89-18	2.7b-d	0.3	LMS89-19	2.2b-f	0.1	LMS89-30	2.0b-f	0.1
LMA89-45	2.5b-e	0.4	LMS89-25	2.2b-f	0.1	LMS89-55	2.0b-g	0.1
LMM89-2	1.2fg	0.1	LMS89-32	2.2b-f	0.0	LMS89-31	1.9b-g	0.2
LMM89-1	0.7g	0.1	LMS89-33	2.2b-f	0.1	BL87-9	1.9b-g	0.1
Mean	2.9	0.2	LMS89-35	2.2b-f	0.1	Leroy	1.9c-g	0.1
			LMA89-43	2.2b-f	0.1	LMS89-5	1.9d-g	0.3
			LMS89-51	2.2b-f	0.1	LMS89-29	1.9d-g	0.1
			LMS89-52	2.2b-f	0.1	LMS89-17	1.8d-g	0.2
			LMS89-53	2.2b-f	0.1	LMM89-9	1.3e-g	0.2
			LMS89-60	2.2b-f	0.1	Mean	2.2	0.0

[†] Mean expansion rate (mm day^{-1}) of five colony radii within each of three replicates

[‡] Means with the same letter are not significantly different by Tukey's multiple range (HSD) test at the 1% level.

Table 2.18 Conidiospore germ tube extension rate[†] of *Leptosphaeria maculans* isolates on 2% water agar.

Weakly virulent isolates			Virulent isolates					
Isolate	Mean[‡]	SE[§]	Isolate	Mean	SE	Isolate	Mean	SE
AVBL89-23	814.4a	27.8	LMA89-42	282.4i	9.9	LMS89-52	238.5i-l	9.5
LMA89-40	790.3ab	18.6	LMS89-19	272.4i	11.7	LMM89-82	237.8i-l	11.0
LMA89-79	785.3ab	20.3	LMM89-48	269.5i	10.5	LMS89-33	237.2i-l	8.3
BL86-22(4)	757.3a-c	18.1	VBL89-72	269.2i	12.0	LMS89-51	236.7i-l	10.1
9LMA89-1	752.4a-d	18.5	VBL89-20	266.1i	10.4	LMS89-17	235.8i-m	11.6
LMS89-54	745.3b-d	18.7	LMS89-30	262.4i-j	10.9	BL87-10	232.3i-m	7.5
LMA89-78	739.7b-d	21.8	LMA89-43	262.3i-j	9.0	LMS89-25	232.1i-m	12.0
LMA89-45	731.4b-e	21.3	BL87-9	262.3i-j	11.2	LMA89-81	231.0i-m	10.4
AVBL89-96	709.6e-c	31.6	LMS89-7	259.7i-j	11.4	LMA89-64	229.5i-n	9.4
LMM89-11	709.5e-c	23.0	LMS89-32	259.6i-j	9.4	LMA89-3	229.1i-n	10.1
LMS89-18	692.5d-f	22.4	LMS89-75	256.2i-k	8.5	LMS89-61	228.7i-n	7.4
LMS89-34	641.0f-g	9.1	LMA89-44	253.1i-l	9.3	LMS89-27	227.2i-n	6.8
LMS89-26	641.0f-g	19.1	VBL89-14	253.1i-l	10.3	LMS89-35	222.8i-n	8.7
LMM89-2	619.0g	24.2	LMS89-29	252.6i-l	6.2	LMM89-9	222.8i-n	8.8
LMM89-1	517.6h	15.9	LMS89-31	252.5i-l	7.8	LMS89-22	217.4i-n	9.9
LMA89-41	509.8h	10.5	LMA89-77	252.1i-l	8.7	LMS89-14	214.8i-n	9.1
LMS89-12	430.0i	16.3	VBL89-95	251.0i-l	13.1	LMM89-8	213.4i-n	8.1
LMS89-24	426.1i	19.8	LMS89-55	250.9i-l	8.9	Leroy	196.7j-n	8.9
LMS89-58	263.8i-j	11.1	VBL89-97	248.1i-l	8.0	LMA89-65	167.4m-n	7.9
LMS89-36	238.5i-l	14.0	LMS89-53	248.0i-l	7.6	LMM89-37	161.2n	5.7
LMM89-5	228.1i-n	12.4	LMS89-60	246.7i-l	8.8	LMM89-4	83.5o	3.0
			LMM89-49	240.2i-l	10.3			

[†] mm day^{-1}

[‡] Mean of three replicates. Means with the same letter are not significantly different by Tukey's multiple range (HSD) test at the 1% level.

[§] Standard error of the mean.

Table 2.19. Pigment production by weakly virulent *Leptosphaeria maculans* in modified Czapek's broth.

	Relative absorbance (405nm)		
Isolate	Mean†	Mean rank‡	S.E.§
LMS89-34	3.9695	257.50a	1.1584
LMA89-78	3.2233	255.50a	0.4774
LMM89-2	2.6520	248.00a	0.5497
AVBL89-23	1.9945	238.50a	0.2885
LMS89-12	1.9810	237.50a	0.3050
AVBL89-96	3.0515	257.00a	0.2812
LMS89-18	1.9833	235.00a	0.3136
LMA89-40	1.7820	234.13a	0.1412
LMA89-45	1.8740	231.50a	0.4188
BL86-22(4)	2.3255	225.50ab	0.9849
LMA89-79	1.4650	218.88ab	0.1374
LMS89-24	1.4820	218.00ab	0.2569
LMA89-41	1.2898	209.00ab	0.1210
LMA89-91	1.5608	206.75ab	0.3641
LMM89-1	1.1920	203.75ab	0.1262
LMS89-54	0.8885	132.38ab	0.3598
LMM89-11	0.9407	131.06ab	0.2015
LMS89-36	0.4765	94.38ab	0.1124
LMS89-58	0.4545	90.00b	0.1118
LMM89-5	0.2835	49.25b	0.1085
Mean	1.7435	183.69	0.0244

† Mean of four replicates.

‡ Mean rank among all isolates tested. Mean ranks with the same letter are not significantly different by Tukey's HSD at the 99% confidence interval.

§ Standard error of mean relative absorbance.

Table 2.20. Pigment production by virulent *Leptosphaeria maculans* in modified Czapek's broth.

	Absorbance (405nm)				Absorbance (405nm)		
Isolate	Mean†	Mean rank‡	S.E.§	Isolate	Mean	Mean rank	S.E.
LMM89-9	1.5050	219.75ab	0.2026	LMS89-19	0.5108	99.75abc	0.1019
LMS89-26	1.3568	211.75abc	0.1699	LMS89-60	0.4890	96.38abc	0.0598
VBL89-14	0.9000	169.88abc	0.1951	LMA89-43	0.4715	89.50abc	0.1276
VBL89-14	0.7865	169.38abc	0.0523	LMS89-31	0.4383	89.50abc	0.1313
LMS89-17	0.7677	166.00abc	0.0845	LMS89-61	0.4717	89.33abc	0.0766
LMS89-35	0.6770	145.88abc	0.0614	LMM89-8	0.4495	88.63abc	0.1166
LMS89-30	0.6658	143.88abc	0.0722	LMS89-29	0.5148	88.50abc	0.2354
LMM89-6	0.7295	143.75abc	0.1603	LMS89-55	0.4467	86.67abc	0.1296
LMA89-77	0.6537	141.17abc	0.0815	BL87-9	0.4088	75.63abc	0.0941
BL87-10	0.6353	135.38abc	0.0482	LMM89-3	0.4030	75.38abc	0.0982
LMS89-27	0.6220	134.25abc	0.0769	LMS89-33	0.3994	73.56abc	0.0543
VBL89-95	0.6015	125.38abc	0.1357	VBL89-20	0.3900	72.63abc	0.1071
LMA89-44	0.6010	123.50abc	0.1152	LMS89-51	0.3765	70.00abc	0.1283
LMM89-82	0.5888	123.25abc	0.0504	VBL89-97	0.3718	65.25abc	0.0434
LMS89-75	0.6238	120.38abc	0.1867	LMS89-22	0.3568	65.00abc	0.1011
LMM89-7	0.5693	112.25abc	0.1638	LMS89-25	0.3667	64.33abc	0.0741
LMS89-37	0.5385	109.25abc	0.0437	LMS89-32	0.3720	64.33bc	0.0612
LMA849-42	0.5300	108.88abc	0.0541	LMS89-14	0.3095	54.38c	0.1010
LMS89-52	0.4908	102.75abc	0.1446	LMM89-4	0.3210	53.50c	0.0790
VBL89-72	0.5045	101.75abc	0.1453	LMM89-48	0.2993	53.00c	0.1220
				LMS89-53	0.2838	48.38c	0.0921
				LMA89-65	0.1488	18.25c	0.0401
				Mean	0.5547	106.59	0.0243

† Mean of four replicates. ‡ Mean rank among all isolates tested. Ranks with the same letter are not significantly different by Tukey's standardized (HSD) Range test at the 99% confidence level.

§ Standard error of relative absorbance.

Table 2.21. Image analysis of pigment production by weakly virulent *Leptosphaeria maculans* isolates in modified Czapek's agar.

Isolate	PI[†]	Rank[‡]	S.E.[§]
LMM89-1	58.7	4.14	3.4
LMM89-2	43.7	1.25	7.8
LMM89-11	64.3	28.59	1.9
LMS89-12	50.3	9.15	9.7
LMS89-18	50.0	9.16	11.2
LMS89-24	64.3	22.49	1.8
LMS89-34	38.3	7.07	8.9
LMS89-36	41.7	25.63	12.2
LMA89-40	50.0	10.60	9.5
LMA89-41	49.3	13.79	9.2
LMA89-45	41.3	23.34	11.9
LMS89-54	53.7	28.09	12.8
LMS89-58	30.3	1.03	1.2
LMA89-78	66.3	32.88	1.5
LMA89-79	51.7	65.17	11.1
LMA89-91	41.0	22.62	11.5
AVBL89-23	49.3	5.58	9.7
AVBL89-96	53.3	225.35	12.8
BL86-22(4)	35.7	1.79	6.2
BL4-wv	66.0	57.05	2.0
Mean	29.74	49.97	10.76

[†] Mean pigment index of three replicates.

[‡] Mean rank among all isolates tested.

[§] Standard error of mean relative absorbance.

Table 2.22. Image analysis by virulent *Leptosphaeria maculans* isolates in modified Czapek's agar.

Isolate	PI†	Rank‡	S.E.§	Isolate	PI	Rank	S.E.
LMM89-3	23.3	1.00	6.2	LMM89-48	23.3	1.00	6.2
LMM89-4	23.3	1.00	6.2	LMM89-49	33.3	1.19	3.9
LMM89-5	29.3	1.01	0.9	LMS89-51	23.3	1.00	6.2
LMM89-6	23.3	1.00	6.2	LMS89-52	23.3	1.00	6.2
LMM89-7	31.3	1.10	2.0	LMS89-53	23.3	1.00	6.2
LMM89-8	23.3	1.00	6.2	LMS89-55	23.3	1.00	6.2
LMM89-9	45.0	1.57	8.1	LMS89-60	36.7	2.16	7.2
LMS89-14	30.7	1.06	1.5	LMS89-61	35.3	1.63	5.9
LMS89-17	23.3	1.00	6.2	LMA89-64	36.0	1.83	6.6
LMS89-19	28.0	1.00	1.7	LMA89-65	37.7	3.79	8.2
LMS89-22	23.3	1.00	6.2	LMS89-75	23.3	1.00	6.2
LMS89-25	30.0	1.02	1.0	LMA89-77	32.3	1.17	3.0
LMS89-26	47.3	8.34	10.5	LMA89-81	36.3	1.93	6.9
LMS89-27	29.7	1.02	0.9	LMM89-82	32.7	1.18	3.3
LMS89-29	28.3	1.01	1.5	VBL89-95	23.3	1.00	6.2
LMS89-30	23.3	1.00	6.2	VBL89-14	23.3	1.00	6.2
LMS89-31	27.3	1.00	2.3	VBL89-72	35.0	1.56	5.6
LMS89-32	23.3	1.00	6.2	VBL89-20	34.0	1.23	4.6
LMS89-33	23.3	1.00	6.2	VBL89-97	28.7	1.00	1.2
LMS89-35	32.0	1.17	2.6	BL87-9	23.3	1.00	6.2
LMM89-37	31.0	1.10	1.7	BL87-10	27.7	1.00	2.0
LMA89-42	23.3	1.00	6.2	Leroy	33.0	1.19	3.6
LMA89-43	32.7	1.00	13.0	LMG90-112	31.7	1.11	2.3
LMA89-44	29.0	1.01	1.0	LMA91-117	23.3	1.00	6.2
				LMA90-3356	27.0	1.00	2.6
				Mean	28.9	1.33	0.16

† Mean pigment index of three replicates. ‡ Mean rank among all isolates tested.
§ Standard error of mean relative absorbance.

Figure 2.1. (top). Humidity chamber for incubation of *Brassica napus* seedlings after cotyledon inoculation with *Leptosphaeria maculans*.
(bottom). Detail of inoculated *Brassica napus* seedlings in humidity chamber. Note that flats are covered during incubation. Water vapour in the background was supplied by two ultrasonic humidifiers exhausting into the chamber.

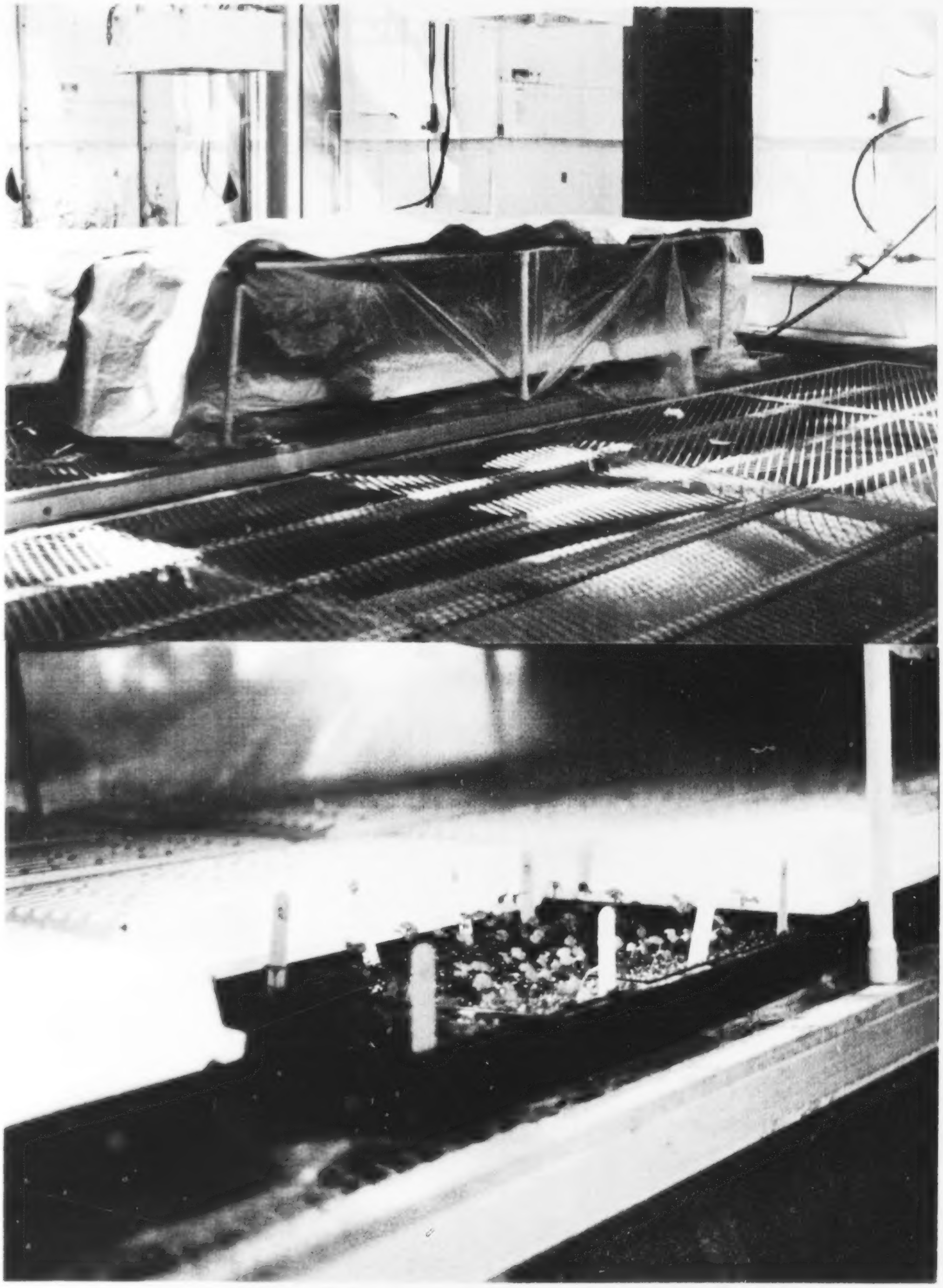
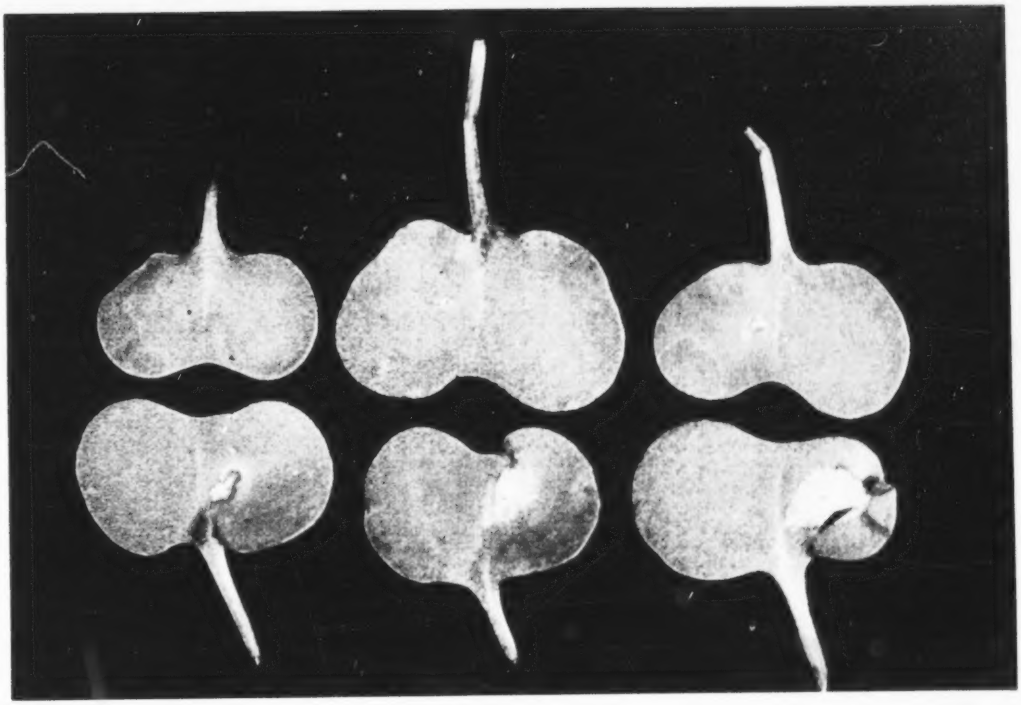


Figure 2.2. Range of interaction phenotypes (IP) obtained by inoculation of *Leptosphaeria maculans* isolates onto *Brassica napus* cotyledons. Phenotypes were rated as follows:
Top row (left to right): IP0, IP1, IP3.
Bottom row: (left to right): IP5, IP7, IP9.



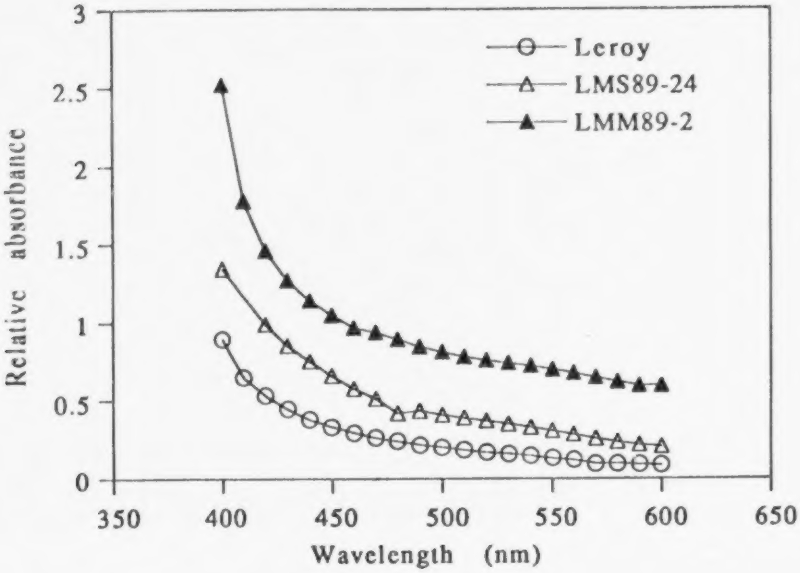


Figure 2.3. Visible range absorbance spectra of culture filtrates for three representative *Leptosphaeria maculans* isolates grown in modified Czapek's broth. Reference samples were filtrates of ininoculated medium.

Figure 2.4. Weakly virulent strain of *Leptosphaeria maculans* (isolate LMS89-54). Nine day-old colony growing on V-8 juice agar medium. Note aerial mycelium, sparse sporulation and pigmented agar.

Figure 2.5. Nine day-old colony of aggressive strain on V-8 juice agar. Note sparse mycelium and profuse sporulation. Also note sector (arrow).

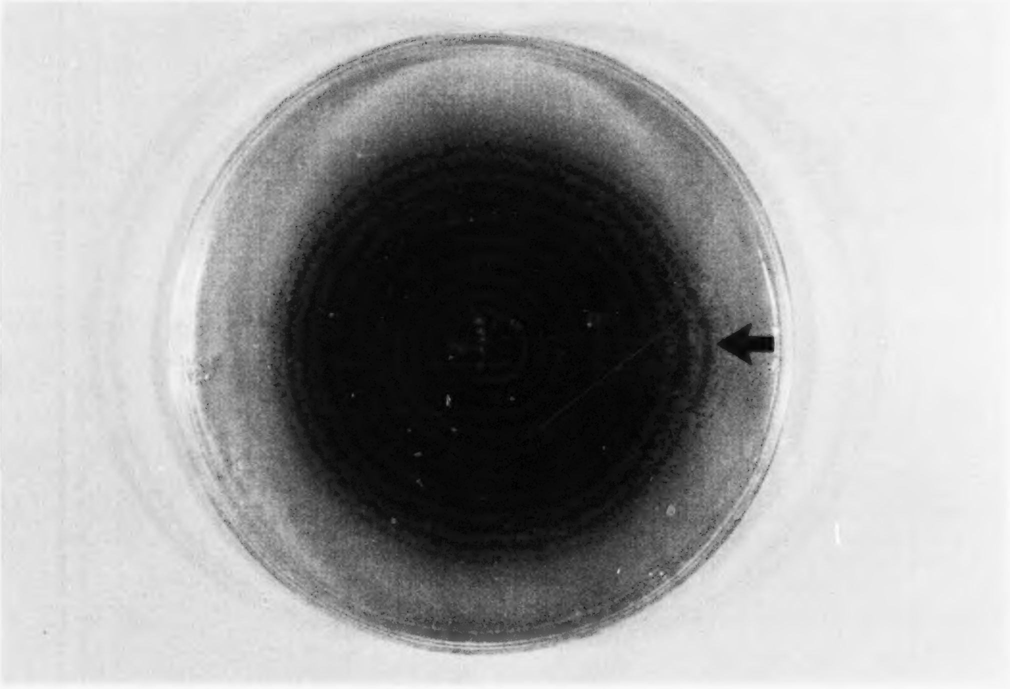
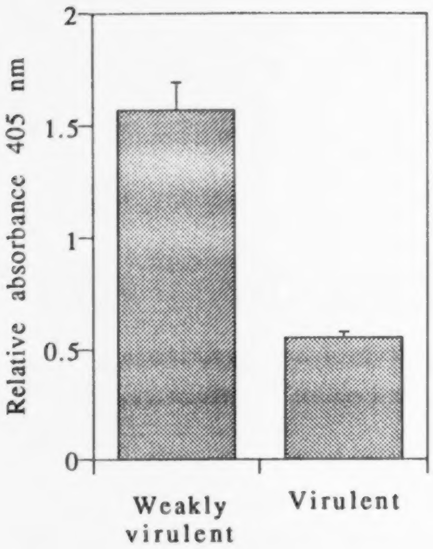


Figure 2.6. Effect of virulence type on relative absorbance of culture filtrates from *Leptosphaeria maculans* isolates. Vertical bars indicate standard errors.

Figure 2.7. Effect of virulence on pigment production by *Leptosphaeria maculans* in modified Czapek's broth.


2
1.5
1
0.5
0
Relative absorbance 405 nm
Weakly virulent
Virulent

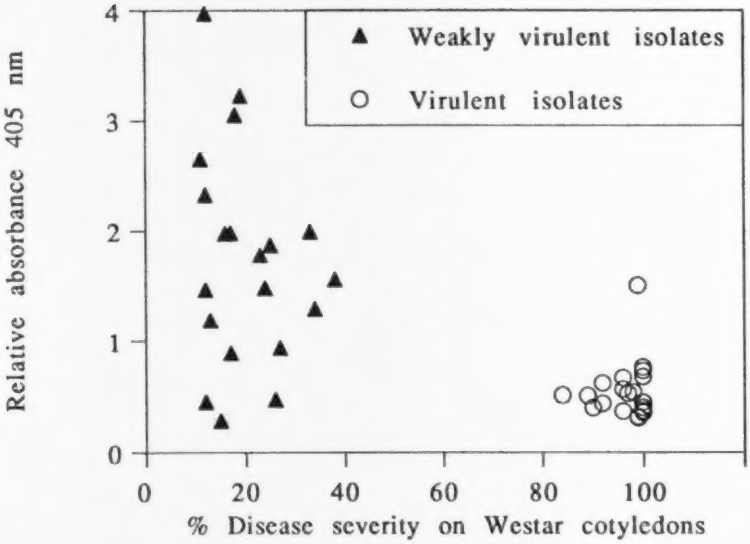
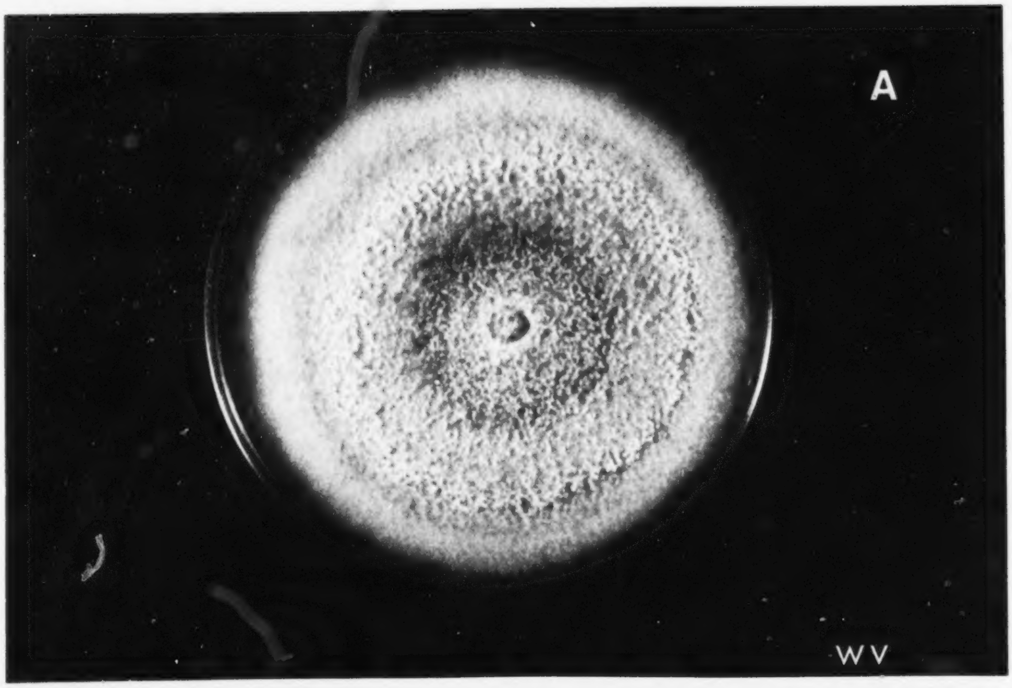

4
3
2
1
0
Relative absorbance 405 nm
▲ Weakly virulent isolates
○ Virulent isolates
0
20
40
60
80
100
% Disease severity on Westar cotyledons

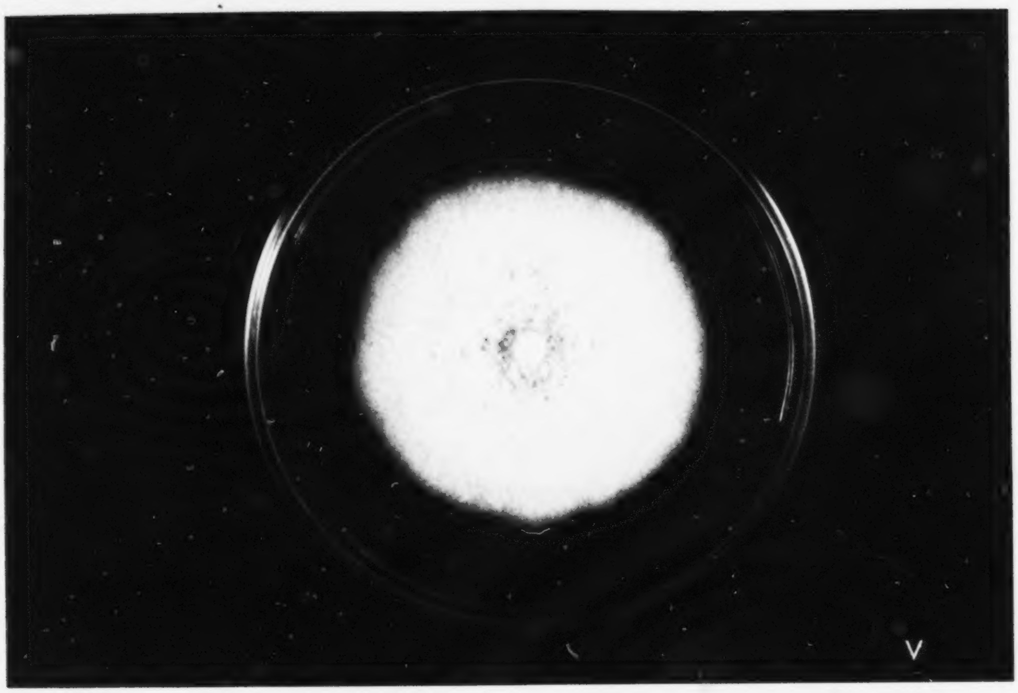
Figure 2.8. Quantification through image analysis of pigment production by weakly virulent (WV) and virulent (W) isolates of *Leptosphaeria maculans*.

A. *L. maculans* colonies on CZY agar prior to removal of cellophane membrane.

B. Digitized images of pigmented agar.

C. Density slice classification of digitized images.


A
WV


V

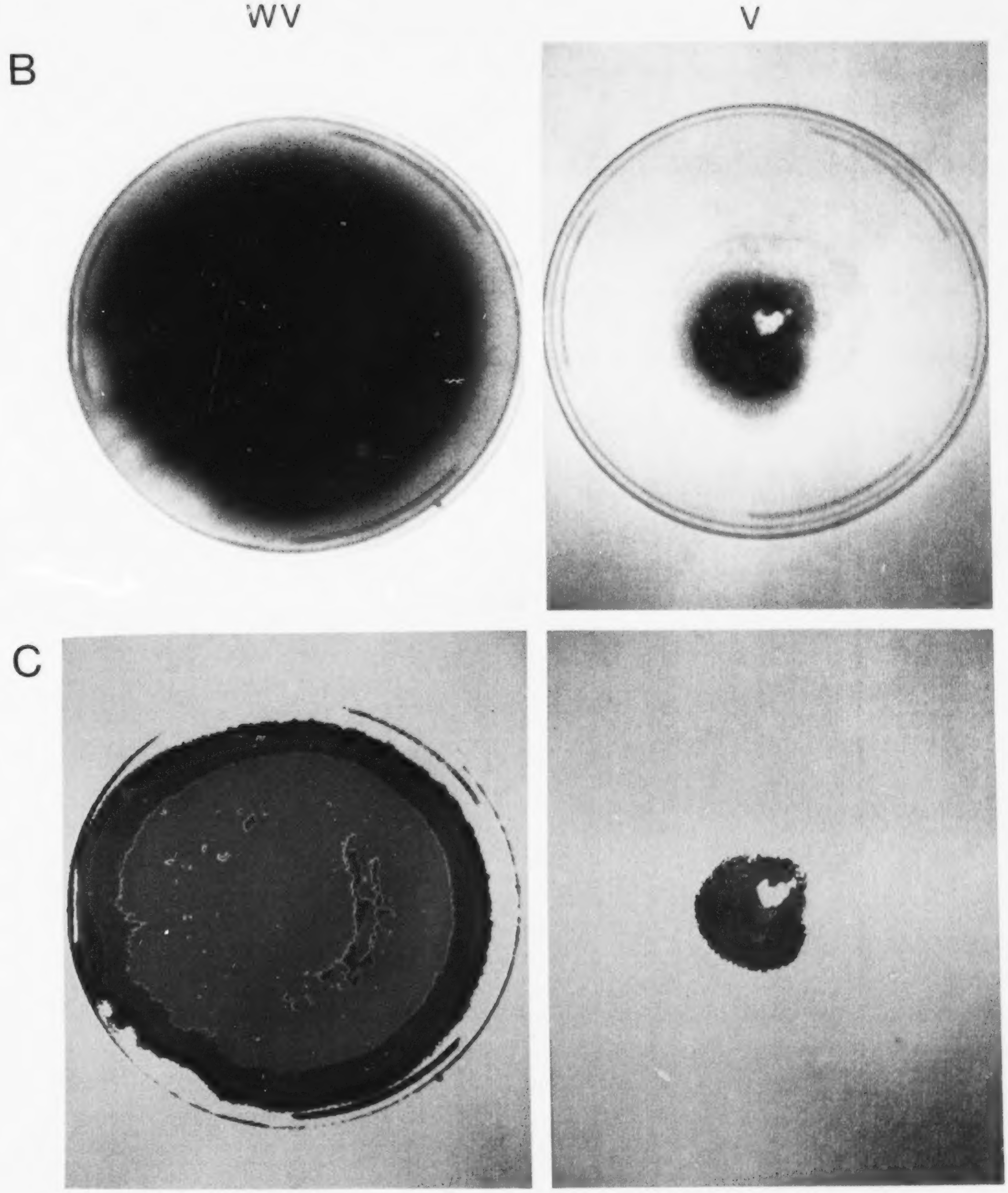
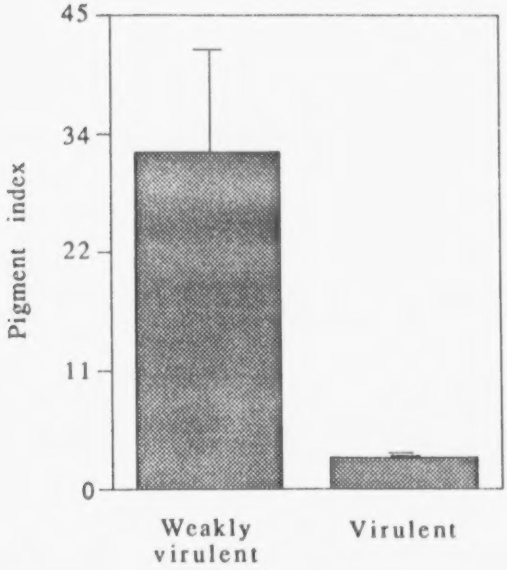

WV
V
B
C

Figure 2.9. Effect of *Leptosphaeria maculans* isolate virulence type on pigment index. Vertical bars indicate standard errors.

Figure 2.10. Relationship between virulence of western Canadian isolates of *Leptosphaeria maculans* and pigment index.


45
34
22
11
0
Pigment index
Weakly virulent
Virulent

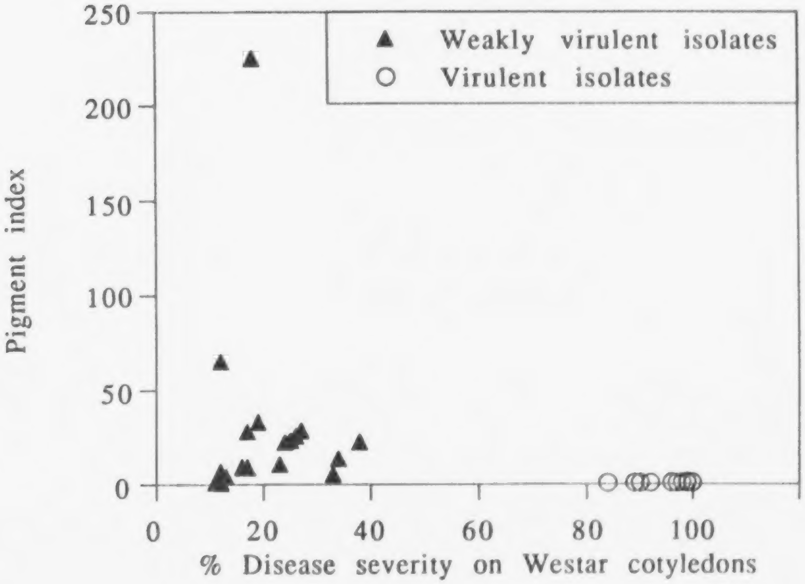
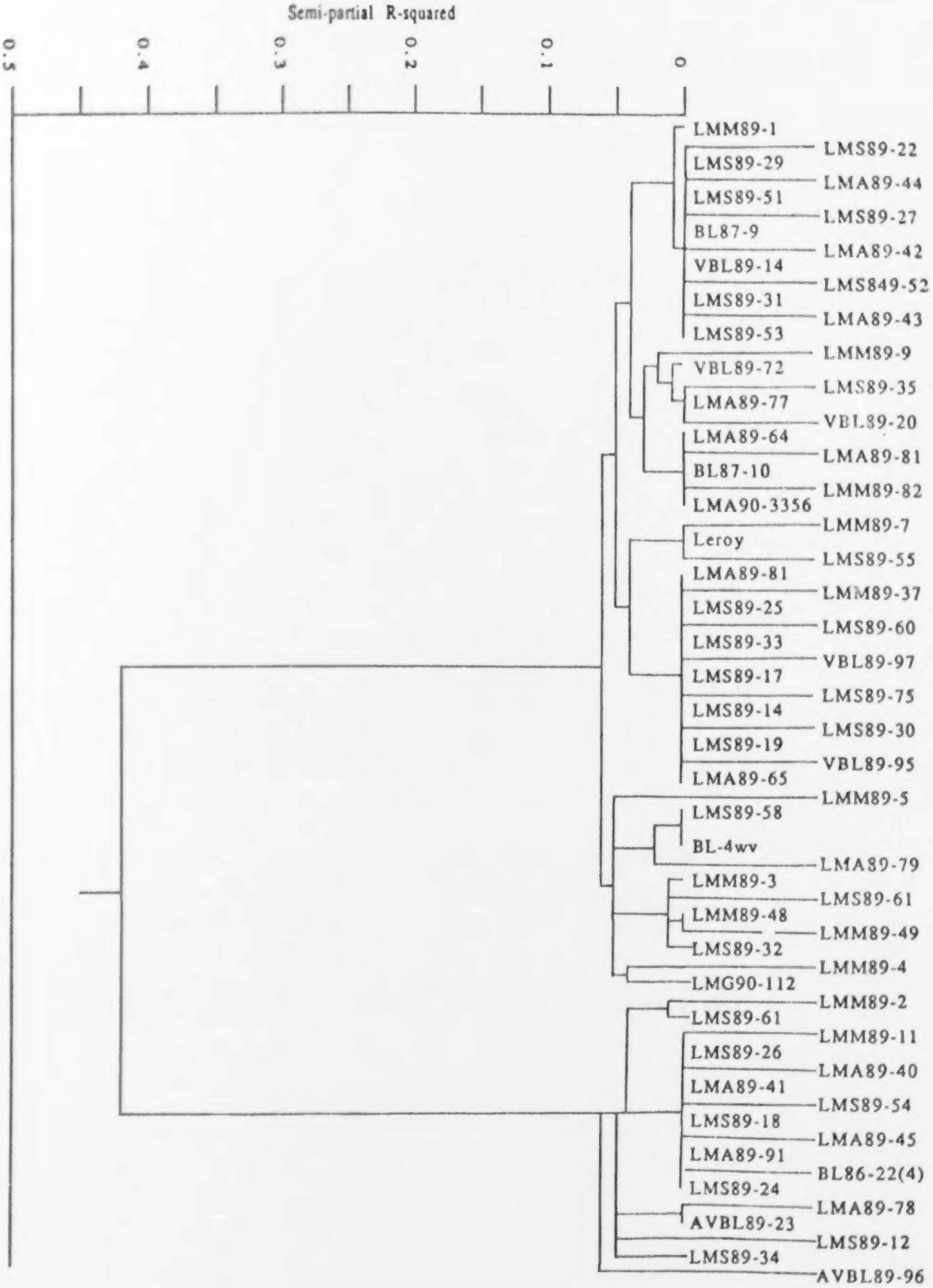

250
200
150
100
50
0
Pigment index
0
20
40
60
80
100
% Disease severity on Westar cotyledons
▲ Weakly virulent isolates
○ Virulent isolates

Figure 2.11. Dendrogram showing *Leptosphaeria maculans* isolates grouped by Ward's clustering method using the variables virulence on cotyledons of *Brassica napus* cultivars Westar, Glacier, Quinta and Maluka; colony morphology on V8A; growth rate on V8A and CZY; conidiospore germ-tube extension rate; pigment production in CZY broth; and pigment production in CZY agar.


Semi-partial R-squared
0.5
0.4
0.3
0.2
0.1
0
LMM89-1
LMS89-22
LMS89-29
LMA89-44
LMS89-51
LMS89-27
BL87-9
LMA89-42
VBL89-14
LMS849-52
LMS89-31
LMA89-43
LMS89-53
LMM89-9
VBL89-72
LMS89-35
LMA89-77
VBL89-20
LMA89-64
LMA89-81
BL87-10
LMM89-82
LMA90-3356
LMM89-7
Leroy
LMS89-55
LMA89-81
LMM89-37
LMS89-25
LMS89-60
LMS89-33
VBL89-97
LMS89-17
LMS89-75
LMS89-14
LMS89-30
LMS89-19
VBL89-95
LMA89-65
LMM89-5
LMS89-58
BL-4wv
LMA89-79
LMM89-3
LMS89-61
LMM89-48
LMM89-49
LMS89-32
LMM89-4
LMG90-112
LMM89-2
LMS89-61
LMM89-11
LMS89-26
LMA89-40
LMA89-41
LMS89-54
LMS89-18
LMA89-45
LMA89-91
BL86-22(4)
LMS89-24
LMA89-78
AVBL89-23
LMS89-12
LMS89-34
AVBL89-96

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Chapter 3

Agglutination response of conidiospores from aggressive and non-aggressive isolates of *Leptosphaeria maculans*.

Introduction

Lectins are proteins or glycoproteins of non-immune origin which bind specific carbohydrate moieties (Goldstein et al. 1980, Lis & Sharon 1986, Sharon & Lis 1993). Because of their ability to bind carbohydrates, lectins have successfully been used to characterize the cell-surface carbohydrates of various fungi. A simple method of characterizing cell-surface carbohydrates of fungi is differential agglutination of cells suspended in lectin solutions.

Agglutination response of fungal spores has been used to differentiate among fungi at the species and *forma speciales* level. Kleinschuster & Baker (1974) found differences in cell-surface glycoconjugates between *Fusarium solani* and *F. avenaceum*. Similarly, Cristinzio et al. (1988) were able to differentiate *F. oxysporum* and *F. solani* from six other *Fusarium* species on the basis of macro- and microconidial agglutination response mediated by Concanavalin A and *Helix pomatia* agglutinin. Sugar residues specific to *Helix pomatia* agglutinin, Concanavalin A and wheat germ agglutinin were found to vary quantitatively between *F. oxysporum* f.sp. *radicis-lycopersici* and *F. o. lycopersici* (Boyer & Charest 1989).

Lectins are useful tools for exploring symbiotic and pathogenic interactions between microbes and their hosts since cell-surface carbohydrates may be determinants of specificity (Aronson 1981, Bonfante-Fasolo & Perotto 1986, Chaboud & Lalond 1983, Sharon & Lis 1993). The location of cell-surface lectin-binding macromolecules have been shown to differ markedly between compatible and incompatible strains of the ectomycorrhizal symbiont *Laccaria bicolor* (Maire) Orton (Lei et al. 1991). Pendland & Boucias (1986) showed that galactose-specific insect lectins bind to cell surface glucan residues found in several entomogenous fungi, and postulated that microbial surface carbohydrates of mycelial insect pathogens are matched by insect lectin specificities. A β-glucan purified from cell walls of *Phytophthora megasperma var. sojae* and a glucan isolated from brewer's yeast extract have been found to induce phytoalexin accumulation in *Phaseolus vulgaris* and

Solanum tuberosum (Cline et al. 1978). Callow (1977) assigned lectins a role in gene-for-gene specificity. He suggested that lectins and their corresponding glucans form a hybrid molecule which in turn signals induction of defense mechanisms, such as hypersensitive response and phytoalexin accumulation.

It has been recognized since early in this century that at least two strains of the phytopathogenic ascomycete *Leptosphaeria maculans* (Desmaz.) Ces. et de Not. parasitize oleiferous *Brassica napus* L. Severe stem cankers result when susceptible *B. napus* genotypes are challenged with the more aggressive of these strains, while the non-aggressive strain causes only mild lesions (Gugel & Petrie 1992, Gabrielson 1983, Williams 1992). In addition to pathogenicity, the two strains can be differentiated on the basis of expression of a number of phenotypes, including electrophoretic karyotype (Taylor et al. 1991), isozyme banding patterns (Hanacziwskyj & Drysdale 1984, Hill et al. 1984), in vitro production of phytotoxins (Koch et al. 1989), pigment production (Delwiche 1980), and morphological parameters (Pound 1947). If the hypothesis of Callow (1977) concerning the mediation of specificity in plant-pathogen interactions by cell-surface glucan residues holds, then it follows that conidia of the aggressive strain of *Phoma lingam* (Tode ex Fr.) Desm. (the conidial state of *L. maculans*) possess cell-surface glycoconjugates which differ from those of the non-aggressive strain. The purpose of this investigation, therefore, was to determine whether cell-surface carbohydrates represent a means of differentiating among *L. maculans* isolates or strains, as determined by spore agglutination mediated by a set of lectins with differing carbohydrate specificities.

Materials and methods

Fungal isolates and culture conditions. Aggressive (LMM89-7, LMS89-22 and Leroy) and non-aggressive (LMS89-18, LMA89-40 and LMA89-41) single conidiospored *L. maculans* isolates were used in all experiments. The aggressive isolate "Leroy" was provided by Dr. G.A. Petrie, Agriculture Canada, Saskatoon. Pure cultures were stored in liquid nitrogen until ready for use. The isolation and cryogenic storage described in Chapter 2 were used.

Cultures of *L. maculans* revived from cryogenic storage were subcultured once before use. Isolates were grown on 2% (w/v) V-8 juice agar

(V8A) (200 ml L^{-1} V-8 juice, 50 mg L^{-1} rose bengal and 3 g L^{-1} $CaCO_3$) until a lawn of sporulating pycnidia was produced (7 to 10 days). Unless otherwise noted, all reagents were purchased from Fisher Scientific Co. (Fair Lawn, New Jersey) or J.T. Baker Chemical Co. (Phillipsburg, New Jersey). Colonies were grown under cool white florescent tubes (ca. 170 µE m^{-2} sec^{-1}) with 16 hrs of light at ambient temperature (ca. 23° C). Conidiospores were harvested by flooding actively sporulating cultures with approximately 10 ml of sterile PBS (50 mM phosphate buffered saline, pH 6.8). Agar plates were then allowed to stand for a few minutes, after which they were gently rubbed with a glass rod. The resulting suspensions were passed through funnels lined with five layers of sterile gauze. This process removed most of the mycelium and free pycnidia from the suspensions. Conidiospores were washed 3× by centrifugation (15 min. at 1000 × g) in cold PBS. All spore harvest and washing operations were carried out under axenic conditions.

Fusarium acuminatium (isolate K.51.4) and *F. avenaceum* (isolate N.15.6) were included in this study as positive controls of lectin-mediated spore agglutination. These isolates were provided by Dr. J.P. Tewari, Department of Plant Science, University of Alberta. *Fusarium* spp. cultures were grown in 125 ml of CMC medium (Booth 1971) composed of 15 g L^{-1} carboxymethylcellulose, (Hercules Powder Co., Wilmington, Delaware), 1 g L^{-1} each of NH_4NO_3, KH_2PO_4, and yeast extract (Difco), and 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$ on a rotary shaker at 200 RPM. Broth cultures of *Fusarium* spp. isolates were maintained until large numbers of microconidia were observed in the medium (7-10 days). *Fusarium* microconidia were harvested by passing broth cultures through five layers of sterile gauze to remove macroconidia and hyphae. Harvest and washing procedures were otherwise identical to those used for *L. maculans* conidia.

Lectins. The lectins used in this study were *Ulex europaeus* agglutinin (UEA-I), wheat germ agglutinin (WGA), Concanavalin A from *Canavalia ensiformis* (Con A) *Helix pomatia* agglutinin (HPA), peanut agglutinin (PNA), Osage orange agglutinin (MPA), and faba bean agglutinin (VFA). All lectins were obtained from Sigma Chemical Co. (St. Louis, Mo.). Lectins and their affinities are listed in Table 3.1. Lectins were solubilized in 50 mM PBS, pH 6.8. The Con A co-factors (0.5 mM Ca^{2+}, 0.5 mM Mg^{2+} and 0.5 mM Mn^{2+}) were added as chloride salts. Solubilized lectins were stored at -12° C until ready for use.

Preparation of erythrocytes. The lectins used in this study are known to agglutinate rat red blood cells (Bird 1954, Creger & Gifford 1952, Sumner & Howell 1936). Therefore, washed rat erythrocytes (male Sprague Dawley) were used as controls to confirm lectin activity. Freshly exanguinated blood was filtered through gauze to remove large clots, followed by centrifugation at 1000 × g for 20 minutes and removal of the supernatant. The pellet was resuspended and washed three times by centrifugation (20 minutes each at 1000 x g) in cold 50 mM PBS. Erythrocyte concentration was adjusted to 2% (v/v) in 50 mM PBS after the last centrifugation.

Lectin agglutination assays. Lectins were scored for their ability (+) or inability (-) to agglutinate pycnidiospores of *L. maculans* and *Fusarium* microconidia. The concentration of *L. maculans* and *Fusarium* spp. spores were set with a hemocytometer (American Optical Corporation, Buffalo NY, Cat. No. 1492) to produce a final concentration of 1 x 10^7 spores ml^{-1} in 50 m M phosphate-buffered saline (PBS), pH 6.8. Multiple hemocytometer fields were counted to ensure the accuracy of spore concentrations.

Agglutination of spores or rat erythrocytes suspended in lectin solution was carried out in 100 µl droplets placed onto the bottom of a 25 x 100 mm Petri plate (Ball 1990). Droplets were separated by a grid drawn onto the bottom of each Petri plate with a grease pencil to prevent accidental merging of droplets. Lectin droplets of desired concentration were placed within each space in the grid, after which spore suspensions were added, starting with the droplets with the lowest agglutinin concentration. To prevent evaporation of the droplets, Petri plates were placed into plastic tubs lined with moist paper towels which were in turn covered with plastic film. Suspensions were incubated in darkness at 21 °C. Observations were made 30 min., 24 and 48 hrs. after each experiment was initiated. Suspended cells were easily visible when droplets were viewed through an inverted compound microscope. All spore × lectin combinations were included on a single Petri plate, and at least four replicate plates were prepared.

Agglutination of conidiospores with crude plant extracts. Crude extracts plant tissue were prepared according to the method of Kojima & Uritani (1974) from the *B. napus* blackleg-resistant cvs. Glacier, Jet Neuf, Maluka, the susceptible *B. napus* cultivar Westar, *B. juncea* L. cv. Cutlass (resistant) and *Lycopersicon esculentum* cv. Starfire (non-host). Plants were grown in a soil-free growth medium in 6 cm pots. Plants were maintained in a greenhouse at approximately 21° C, under a 16 h photoperiod supplemented with 400-W high pressure sodium lamps. All aerial parts of *Brassica* and tomato plants at approximately the 4-6 leaf stage were used for preparation of extracts. Extracts were prepared by homogenizing 250 g of host tissue in 250 ml 0.02 M KCl containing 0.5 g ascorbic acid and 25 g polyvinylpolypyrrolidone (Sigma, Cat No. P-6755). The homogenized mixture was filtered through multiple layers of gauze and centrifuged (8256 × g) for 30 min. The supernatant was removed, passed through Whatman No. 1 filter paper, and stored at -12° C until required. Plant extracts were tested against pycnidiospore suspensions of isolates LMM89-7, LMS89-18, LMS89-22 and LMA89-41. This was done by combining spore suspensions (10 µl, 1×10^7 conidia ml^{-1} final concentration) with undiluted extracts (10 µl) in Petri plates as described with respect to the lectin agglutination assay. Con A (250 µg ml^{-1}) was included as a control. Four plates, each containing all treatment combinations were prepared, and the entire experiment was replicated three times. Incubation conditions were the same as those described above.

Results

Lectin agglutination assays. All lectins except PNA were able to agglutinate washed rat erythrocytes in 50mM PBS after 2 hours incubation at 21° C at a concentration of 250 µg ml^{-1} (Table 3.2). The morphology of erythrocyte aggregates ranged from loose groups of individual cells (Figure 3.1) to amorphous clumps (Figure 3.2), and varied with the type and concentration of the lectin in question. At no time did washed erythrocytes form aggregates in the absence of lectin (Figure 3.3).

Microconidia of *F. avenaceum* were agglutinated by Con A (Figure 3.4) in control experiments (Table 3.3). Agglutination of microconidia (1×10^7 ml^{-1}) was visible after 30 minutes incubation in 500 µl ml^{-1} lectin solution. This response was consistent over all replications of the experiment. None of the lectins agglutinated microconidia of *F. acuminatium*.

No agglutination of *L. maculans* conidiospores was observed. The lectins HPA, STA, UEA-I and Con A agglutinated microconidia of *F. avenaceum*, but failed to agglutinate pycnidiospores of *L. maculans*. In a separate experiment, the lectins UEA-I, WGA, Con A, HPA, PNA, MPA and VFA failed to agglutinate conidia of either the aggressive isolate "Leroy" or the weakly virulent isolate LMA89-40.

Agglutination of conidiospores with crude plant extracts. Since no agglutination of *L. maculans* pycnidiospores was observed, it was decided to attempt agglutination using crude plant extracts, should *B. napus* tissues contain an undiscovered lectin specific to *L. maculans*. Unfortunately, conidiospores of *L. maculans* were agglutinated neither by any of the plant extracts, nor by Con A. This was true even after 48 hours incubation.

Discussion

Wall to wall contact between symbiotic or parasitic microbes and their hosts is thought to invoke a recognition mechanism which, in pathogenic systems, triggers defense responses in the host. These may take the form of containment strategies, production of compounds inhibitory to the microbe, or a hypersensitive response (Sequeira 1978). All three of these phenomena have been observed in the interactions between *B. napus* and *L. maculans* (Hammond & Lewis 1987, Mithen & Lewis 1988, Pedras & Séguin-Swartz 1992, Rimmer & van den Berg 1992). The form and extent that these responses take is partially dependent on the *L. maculans* strain involved in the interaction. For example, it has been shown that weakly virulent isolates elicit a more rapid elicitation and accumulation of phytoalexins (Dahiya & Rimmer 1989), and that the morphology of callose and lignin barriers around infected tissue is determined by pathogen isolate (Hammond & Lewis 1987). A mechanism which enables *B. napus* to discriminate between aggressive and non-aggressive strains of *L. maculans* must therefore exist.

Glycoconjugate-lectin hybrid molecules may be determinants of specificity since a large number of configurations are possible under this scheme. If recognition of cell-wall carbohydrates is the arbiter of specificity in plant-pathogen interactions, then the cell-surface carbohydrate moieties present in aggressive isolates of *L. maculans* should differ in some respect from those of the weakly virulent strain. An analogous relationship between *formae speciales* of *F. oxysporum* has been described (Boyer & Charest 1989).

Similarly, different lectin specificities have been found among *Fusarium* species (Kleinschuster & Baker 1974).

The procedures used in this study, however, failed to detect such differences among strains of *L. maculans*. *Fusarium* microconidia and washed rat erythrocytes, however, generally did agglutinate, showing that the lectins were active under the experimental conditions applied in this study. The lack of *L. maculans* conidiospore agglutination is surprising, since the lectins used were specific for glucans commonly found in fungi. Three of these carbohydrates D-glucose, N-acetyl-D-glucosamine, and D-mannose are consistently found in the cell walls of most fungi (Bartniki-Garcia 1968). Furthermore, the chitin-binding lectin STA failed to agglutinate *L. maculans* conidia, even though the presence of chitin is one of the distinguishing characteristics of higher fungi (Agrios 1988). The latter result, however, may be due to the location of chitin in cell walls of fungi, where it usually acts as a structural carbohydrate buried within an amorphous matrix (Aronson 1981), rendering it inaccessible to lectin binding without prior removal of surface layers. The N-acetyl-D-glucosamine binding lectin PNA failed to cause agglutination of erythrocytes or conidia of either *Fusarium* or *L. maculans*. Binding of this lectin is inhibited by the presence of terminal sialic acid residues, and thus will fail to agglutinate human erythrocytes without neuraminidase treatment (Lotan et al. 1975). This may indicate that sialic acid residues are blocking PNA binding sites on the cells tested, although a second N-acetyl-D-glucosamine binding lectin, WGA, also failed to cause agglutination.

Although these results appear to indicate that the carbohydrates specific to the lectins used in this study were absent from the conidiospore walls of *L. maculans*, it is probably more likely that the frequency of these moieties is too low to cause agglutination of pycnidiospores. In light of these findings, a more fruitful approach may be to examine cell-surface carbohydrates by high-resolution cytochemical techniques, such as fluorescein- or gold-conjugated lectins (Bonfante-Fasolo & Perotto 1986, Lei et al. 1991). Such an approach would reveal not only differences in binding specificities of particular lectins, but also morphological variations among isolates with respect to this trait.

Agglutination of fungal spores is a rapid means of determining lectin-binding specificities. It is possible, however, that hyphal or germ-tube cell walls of *L. maculans* differ considerably from those of conidiospores. The mechanism of vegetative wall formation during spore germination has not been studied, but in one of the most common forms of morphogenesis, the germ-tube wall is initially an extension of the spore wall. A feature of this type of germ tube formation, common in many higher fungi such as *Aspergillus* spp., *Melampsora* spp. and *Neurospora* spp., is that it probably involves no fundamental change in cell-wall composition (Bartniki-Garcia 1968). If this is the case in *L. maculans*, then carbohydrate composition of conidiospores should reflect the composition of structures directly involved in infection, namely germ-tubes and hyphae.

It is possible, however, that the lectins chosen to differentiate spores of *L. maculans* isolates were not able to cause differential agglutination. Because of this, crude plant extracts were prepared from tolerant and intolerant host plants, as well as non-host plants. The purpose of this was to ascertain whether as-yet undiscovered lectins existed in *B. napus* which were able to differentially agglutinate *L. maculans* conidiospores from different strains. This approach proved effective in agglutinating conidiospores of *Ceratosistis fimbriata*, a pathogen of sweet potato (Kojima & Uritani 1974). Unfortunately, none of the extracts prepared in this study agglutinated conidia of *L. maculans*. The concentrations of any lectins present in the extract may have been too low to cause such a reaction, were destroyed or inactivated during the preparative steps, or were competitively bound to other carbohydrates in the extracts. It should also be pointed out here that no lectins have been isolated from *Brassica* to date.

In conclusion, it is apparent that the methods used in this study were ineffective in determining whether differences exist among different strains of *L. maculans*. Although these simple methods were effective in differentiating among pathotypes of other fungi, notably *Fusarium*, they proved ineffective when applied to *L. maculans*. It may be, on the other hand, that the avirulent strain of *L. maculans* does not differ substantially from the aggressive strain with respect to cell-surface carbohydrate composition. The differences in aggressivity between the two strains may not be a specificity phenomenon, but simply a difference in the strains' pathogenic ability. For example, the weakly virulent strain of the fungus may not be able to overcome preformed defenses such as epidermal wax, or glucosinolates in the host tissues. Alternately, the aggressive strain may more readily overcome induced host defenses and have, for example, the ability to metabolize phytoalexins.

Table 3.1. Carbohydrate-binding specificities of eight lectins used to characterize spore cell-surface carbohydrates.

Lectin	Major affinities
UEA-I	L(-)Fucose
WGA	N-Acetyl-D-glucosamine
STA	N,N',N"-Triacetylchitotriose
ConA	Sucrose>>N-Acetyl-D-glucosamine
HPA	N-Acetyl-D-glucosamine>>N-Acetyl-D-galactosamine
PNA	N-Acetyl-D-glucosamine
MPA	Mannose>Galactose
VFA	N-Acetyl-D-glucosamine, Glucose, Sucrose, Mannose

Table 3.2. Agglutination response[†] of washed rat erythrocytes[‡] to six lectins

	Lectin						
Lectin concentration (mg ml^{-1})	UEA-I	WGA	ConA	HPA	PNA	MPA	VFA
500.00	+	++	++	++	-	++	++
250.00	+	++	++	+	-	++	++
125.00	+	++	++	-	-	++	++
62.50	-	++	++	-	-	++	++
31.25	-	++	++	-	-	++	++
15.63	-	++	++	-	-	++	++
7.81	-	++	++	-	-	++	+
3.91	-	++	+	-	-	++	-
0.00	-	-	-	-	-	-	-

[†] Response rated visually as ++ (strong agglutination), + (moderate agglutination), or - (no agglutination). Results did not vary among four replicate plates.

[‡] Cells washed 3× and suspended (2% v/v) in 50 mM PBS, pH 6.8.

Figure 3.1. Heavily agglutinated washed rat erythrocytes. Cells were incubated for 2 hours in 7.81 mg ml^{-1} MPA solubilized in 50 mM PBS, pH 6.8. Bar indicates 10 µm.

Figure 3.2. Moderately agglutinated washed rat erythrocytes. Cells were incubated for 2 hours in 7.81 mg ml^{-1} VFA solubilized in 50 mM PBS, pH 6.8. Bar indicates 10 µm.

Figure 3.3. Washed rat erythrocytes incubated for 2 hours in 50 mM PBS, pH 6.8. Note lack of agglutination. Bar indicates 10 µm.

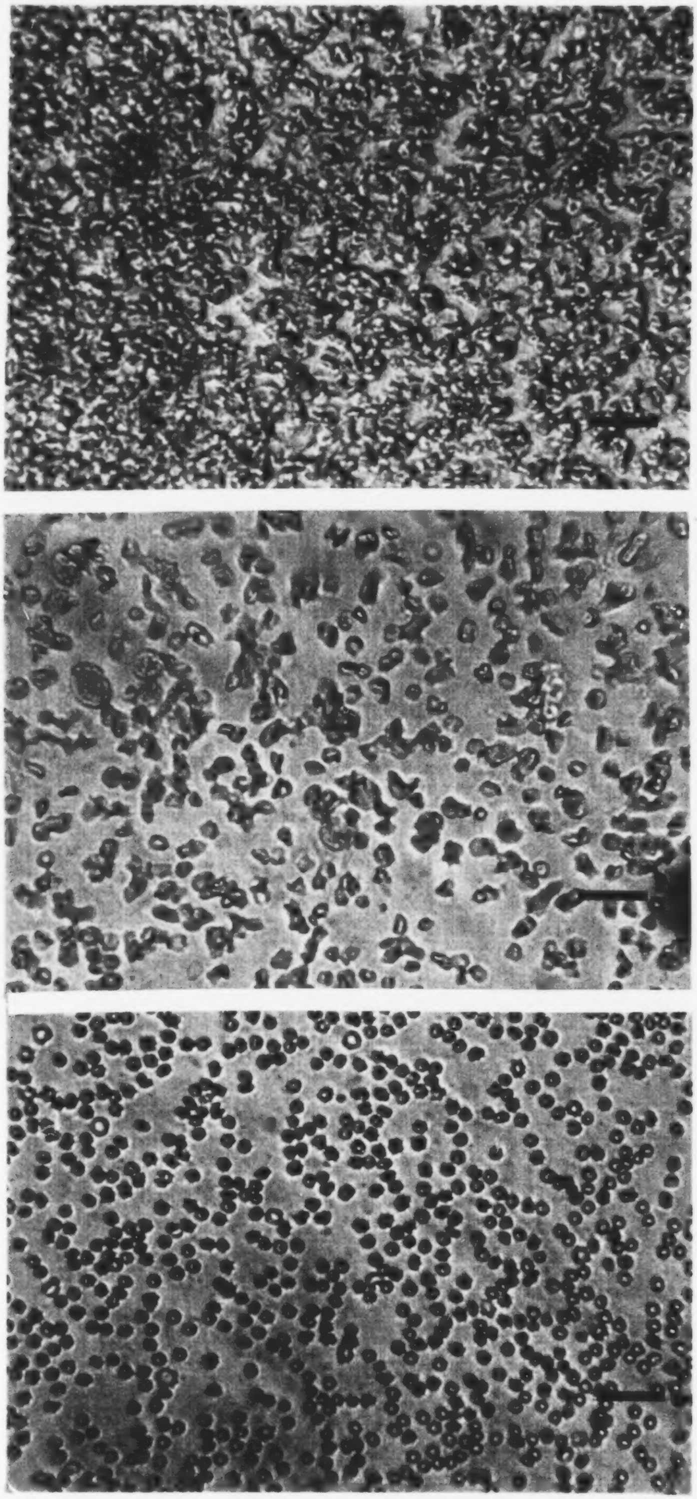
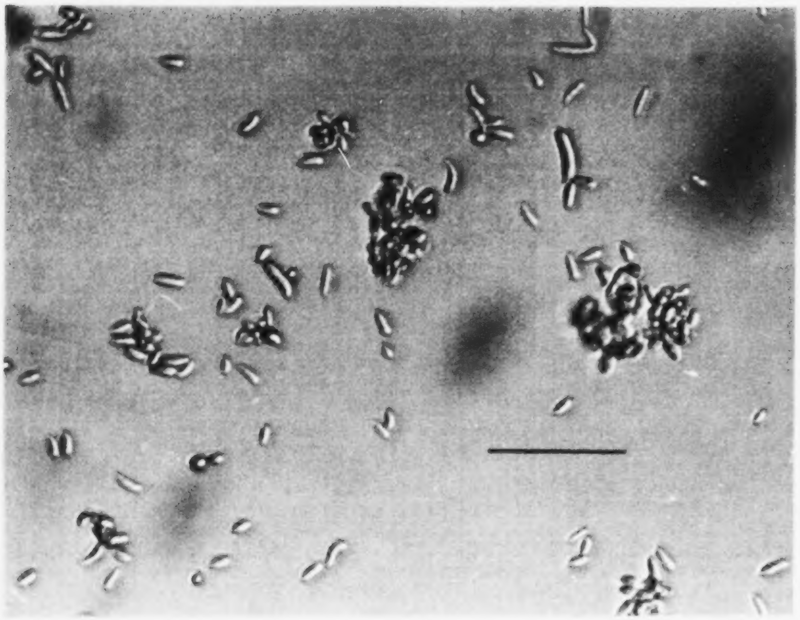


Figure 3.4. Microconidia of *Fusarium avenaceum* agglutinated by 500 mg ml^{-1} Con A in 50 mM PBS, pH 6.8. Bar indicates 500 μm.



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Chapter 4

Summary and Conclusions.

The two strains of *Leptosphaeria maculans* found in western Canada in this study were distinguishable on the basis of colony morphology, germ-tube extension rate and *in vitro* pigment production. One group of isolates produced floccose, rapidly-growing mycelia, few pycnidia, and a brownish pigment in culture. Isolates in the second, larger group had white, sparse, slowly expanding mycelia, abundant pycnidia, and produced little or no pigment. These results are consistent with descriptions of the two strains of the pathogen published previously (Delwiche 1980, Hill et al. 1984, Petrie 1988). These workers reported that isolates with culture characteristics similar to those of isolates in the larger group were aggressive on susceptible cultivars of *Brassica napus*, such as Westar. Isolates with culture characteristics similar to isolates in the first group were found to be non-aggressive in previous studies (Delwiche 1980, Hill et al. 1984, Petrie 1988). These relationships between colony morphology, cultural characteristics and aggressivity are the basis for the division of *L. maculans* into aggressive and non-aggressive strains.

Of the morphological parameters studied, germ-tube extension rate appeared to be most closely correlated with aggressivity. One reason for this correlation may be the short time span and space required to carry out the protocol, thereby presenting less time and spatial heterogeneity for environmental variables to affect the results. The relative reliability of the germ-tube test as well as the speed with which results can be obtained highlight the potential of the method for the rapid determination of isolate pathogenicity (Petrie 1988).

Pathogenicity screening demonstrated that western Canadian isolates conform to the two-strain model of *L. maculans* population structure. In accordance with this model, one group of isolates caused localized necrosis only when inoculated onto cotyledons of the blackleg susceptible cultivar Westar. In contrast, isolates in the second group were unable to accomplish the same on the cotyledons of the differential *B. napus* cultivars Glacier or Quinta. These groups correspond to the pathogenicity groups PG1 and PG2, respectively, as defined by Koch et al. (1989). No other pathotypes were observed, with the exception of isolate LMG90-112, which was able to cause severe lesions on cotyledons on plants of the latter two differential cultivars. This isolate, however, was originally collected in Germany and thus belongs to a geographically distinct *L. maculans* population.

The observed lack of diversity in morphology and pathogenicity characteristics may reflect a corresponding lack of diversity in genotypes among aggressive isolates of *L. maculans* in western Canada. This, in turn, may be explained by a single introduction of the virulent strain into western Canada. Such an introduction would probably have occurred at a time and place near to where aggressive *L. maculans* was first noted; in the mid-1970's in central Saskatchewan (McGee & Petrie 1978).

The uniformity among aggressive *L. maculans* isolates in western Canada implies that screening for resistance of *B. napus* germplasm against a single isolate should produce breeding lines resistant to blackleg across the prairie provinces. Thus, breeding for resistance to blackleg may be greatly simplified by the lack of cultivar-level host specialization of the blackleg pathogen, since a single aggressive isolate should possess all the virulence genes present in the western Canadian *L. maculans* population. Furthermore, results obtained from field screening of *B. napus* germplasm blackleg resistance in any one geographical location in western Canada should be applicable to all canola-growing areas in the prairie provinces. This is true because the uniformity of aggressive western Canadian isolates precludes geographic variation in *L. maculans* pathotype.

The simple pathotype structure of the aggressive *L. maculans* population has the advantage of simplifying introgression of blackleg resistance into *B. napus* germplasm. The presence of only one pathotype of aggressive *L. maculans* means that resistances against multiple physiological races of the pathogen need not be incorporated. This advantage may be nullified, however, by associated vulnerability of western Canadian blackleg resistant cultivars to catastrophic *L. maculans* race change. The resistance to the single western Canadian pathotype of aggressive *L. maculans* does not guarantee resistance to other pathotypes, represented in this study by isolate LMG90-112. It is unlikely, in fact, that many of the spring-type blackleg-resistant *B. napus* cultivars currently registered in Canada are resistant to PG3 or PG4 (Koch et al. 1989), since cultivars developed in Canada were probably not screened against these strains during the selection process. Furthermore, selection of germplasm for resistance solely against Canadian *L. maculans* isolates may have produced a high degree of homogeneity with respect to blackleg resistance among Canadian *B. napus* cultivars. Consequently, pathogen race change via mutation or through the introduction of new pathotypes could conceivably have an effect as devastating as that experienced after the initial spread of PG2. To guard against the occurrence of such an event, blackleg resistance-breeding protocols should include screening of *B. napus* accessions against all pathotypes of aggressive *L. maculans* .

Stringam et al. (1992) have suggested that the putative single-gene resistance in *B. napus* may prove to be stable over time. Major (or single) gene resistances, however, are generally thought to be unstable in comparison to multigene resistances (Parlevleit 1989). This hypothesis of stable single-gene resistance therefore implies the presence of a "strong" vertical resistance gene, in the sense of van der Plank (1968). The matching pathotypes of such "strong" genes are by definition, rare, in order to account for the stability of such resistance systems (Robinson 1976). These matching pathotypes are not rare in the case of blackleg, since PG3 and PG4 isolates are able to attack *B. napus* cultivars resistant to the ubiquitous western Canadian pathotype, PG2. In light of this, any additional resistance genes may become agronomically important should resistances incorporated into current *B. napus* cultivars fail.

Interestingly, a range of reactions was obtained when cotyledons of the Australian blackleg-tolerant cultivar Maluka were challenged with western Canadian isolates aggressive towards Westar. This observation could be explained by polygenic inheritance of resistance, since the continuous distribution of virulence of *L. maculans* isolates with respect to Maluka suggests that resistance in this cultivar is a quantitative trait. Virulence/resistance genes additional to the single (Stringam et al. 1992) or two-gene (Rimmer & van den Berg 1992) systems previously proposed may be present in the *L. maculans/B. napus* pathosystem. These genes may be useful in improving the stability of blackleg resistance in *B. napus*.

If blackleg resistance in *B. napus* is vertical, and if this resistance is controlled by one or two genes combined with low horizontal resistance, then loss of resistance is likely via introduction of or mutation to a matching vertical pathotype (Robinson 1976). Thus, pathogen race change and catastrophic loss of blackleg resistance may be expected in the future. Quantitative-type resistances, such as that present in Maluka-derived *B. napus* accessions, are therefore desirable because of their improved durability in comparison to qualitative resistances. Consequently, a determination should be made as to whether any heritable quantitative resistance can be found in Maluka or in other *B. napus* cultivars.

Any search for such resistance should utilize doubled-haploid derived *B. napus* lines. The wide range of disease severity values obtained when Maluka was challenged with *L. maculans* may have been due to heterozygosity or heterogeneity among Maluka plants with respect to blackleg resistance. One approach to finding quantitative resistance within B. *napus* cultivars would be to screen a number of homozygous lines derived from a single cultivar against a set of *L. maculans* isolates. Lack of specific interactions would indicate quantitative or horizontal resistance.

It is not known whether the non-aggressive strain of *L. maculans* is of the same species as the aggressive strain. Some evidence exists which suggests that the two strains are in fact distinct species (Hassan et al. 1991, Johnson & Lewis 1990, Meyer et al. 1992, Taylor et al. 1991). In particular, attempts at hybrization of aggressive with non-aggressive strains have failed to date (Bonman et al. 1981, Ndimande 1976, Petrie & Lewis 1985, Venn 1979). The possibility of vegetative compatibility between aggressive and non-aggressive strains has, however, not been explored. Successful anastomosis between hyphae of the two strains would suggest that aggressive and non-aggressive pathotypes do not belong to distinct species. Such a finding would also suggest that hyphal fusions among strains of different pathogenicity groups occur in nature, thereby giving rise to novel pathotypes. A relatively simple method of establishing whether anastomosis occurs would be to pair mutually complementary auxotrophic nitrogen-utilization mutants of *L. maculans*. Anastomosis would be indicated by prototrophic growth. This method has been successfully applied to other fungi, such as *Verticillium dahliae* (Puhalla 1979) and *Fusarium oxysporum* (Correll et al. 1987, Strausbaugh 1992).

Further work is also required with respect to the epidemiology and ecology of *L. maculans*. For example, several studies of the dispersal of ascospores have been conducted. Corresponding studies concerning conidiospores have not been done. Little is known about the comparative ecology of aggressive versus non-aggressive strains. Will competition from aggressive strains eventually drive the population of non-aggressive *L. maculans* to extinction, or do less aggressive isolates hold a competitive advantage which esures their survival? It is possible, for example, that non-aggressive isolates are better adapted as saprophytes, rendering them more fit than aggressive isolates in the absence of living host tissue.

The resistance displayed by *B. napus* cv. Westar cultivars may be non-host resistance (i.e. host-pathogen specificity at the species level) as defined by Heath (1981). This type of resistance is almost certainly controlled by a large number of genes, many of which probably code for a number of nonspecific defense reactions. Heath (1981) proposes that many of the elicitors of these non-specific reactions are non-specific secreted or surface-bound moieties common to many pathogens. She supports this hypothesis with the observation that fungal glucans are comparatively non-specific elicitors of phytoalexin accumulation. For example, glucans common to both *Fusarium oxysporum* f. sp. *lycopersici* and *F.* f. sp. *cubense* have been found by Anderson (1980), who showed that purified glucans from *Fusarium* spp. elicited phytoalexin accumulation in bean, a non-host plant.

Similarly, a lack of specificity in conidiospore surface carbohydrates may account for the lack of differential agglutination of *L.maculans* conidia in this study. However, results of this study were inconclusive because lectins used to characterize cell-surface carbohydrates failed to agglutinate *L. maculans* conidia, regardless of strain. This leaves open the possibility that the selected lectins were not specific to any carbohydrates present on the conidiospore surface, or that the required carbohydrates were too sparsely distributed across the fungal walls to cause agglutination.

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